STIMULUS RESPONSE COUPLING IN THE CAROTID BODY: A ROLE FOR METABOLIC SIGNALLING

by

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A thesis submitted to
The University of Birmingham
for the degree of
DOCTOR OF PHILOSOPHY

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The University of Birmingham
January 2013

Abstract

The mammalian carotid body (CB) is the primary sensory organ that responds to a reduction in arterial O_2 tension. Activation of the CB and stimulation of its chemoafferent fibres evokes a series of well characterised cardiovascular and respiratory reflexes that act to restore normal O_2 levels throughout the organism. A role for an elevated CB chemoafferent outflow in the aetiology and progression of a number of cardiorespiratory disease states has been identified, emphasising the importance of understanding the signalling mechanisms required to activate the CB in hypoxia. In this thesis evidence is provided indicating that the CB response to hypoxia is a consequence of a reduction in mitochondrial energy metabolism and stimulation of the liver kinase B 1/AMP activated protein kinase signalling cascade. Questions also remain over the potential for other stimuli to acutely stimulate the CB. Observations described in this investigation suggest that the CB is not directly sensitive to glucose deprivation and that it preserves energy status in these conditions by metabolism of stored glycogen. Finally, chemoafferent outflow is, ultimately, dependent upon neurotransmission and small molecule neuromodulation. This thesis demonstrates a previously uncharacterised key functional role for adenosine derived from extracellular catabolism of ATP in mediating chemoafferent activity.

Acknowledgements

I would like to take this opportunity to thank Prem Kumar, my lead supervisor and mentor, for all his valuable tutelage and guidance over the last three years. His enthusiasm and knowledge of the carotid body and oxygen sensing have been inspirational. Many thanks to David Hauton, my second supervisor, for his important help and advice, especially regarding metabolism. Special thanks to the Postgraduate School at the University of Birmingham for awarding me the A.E. Hills scholarships which allowed me to continue to study for the Ph.D. after the first year; I am extremely grateful to have been given this opportunity. Thank you to Wendy Leadbeater for her help with establishing the carotid body immunohistochemistry technique, Keith Buckler for allowing me to perform some of the experiments at the University of Oxford and to Mark Evans, at the University of Edinburgh, for the development and shipment of the transgenic animals used within this study. A special thank you to Clare Ray; a trusted advisor and friend. Thank you to Andy Coney for his valuable advice on how to improve my research techniques and presentation of data. Thank you to all of the friends I made in the link labs and especially to Fahima Syeda, Annie Stride and Will Rook for helping me to settle in when I first arrived. Thank you to Clare Box, Abu Gassama and Emma Thompson for putting up with me when I needed to talk things through.

Outside of the University I wish to thank my very close friend Jon Pitt for always being there to cheer me up when I needed it most. Thank you to Jeff Hancock, Wayne Mitchell, Rumel Ahmed and Luke Thackery for their friendship and continued encouragement. Many thanks to all of my team-mates and friends at Stourbridge Racketball Club for trying to make me realise that there is more to life than the carotid body.

Finally, and most important to me, I would like to thank my family. Without their unselfish support I would not have been able to complete my thesis. For this I will always owe a considerable debt of gratitude.

List of abbreviations

4-AP	4-aminopyridine
5-HT	5-hydroxytryptophan (serotonin)
8-SPT	8-(p-sulfophenyl) theophylline (adenosine receptor antagonist)
A_2	adenosine receptor 2
ACh	acetylcholine
AChE	acetylcholine esterase
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AICAR	5-aminoimidazole-4-carboxamide ribonucleotide
AMPK	AMP activated protein kinase
AOPCP	α,β-methylene ADP (CD73 inhibitor)
ARC	arcuate nucleus
ASIC3	acid sensing ion channel 3
ATP	adenosine triphosphate
BK_{Ca}	large conductance Ca^{2+} activated K^+ channel
BSA	bovine serum albumin
C57BL/6J	C57 black 6 mouse strain
CA	catecholamine
CAH	carbonic anhydrase
cAMP	cyclic adenosine monophosphate
CB	carotid body
CBS	cystathione β synthase
CCA	common carotid artery
CCCP	carbonyl cyanide m-chlorophenyl hydrazone
CD39	ectonucleoside triphosphate diphosphohydrolyase 1
CD73	ecto-5'-nucleotidase
cGMP	cyclic guanosine monophosphate
ChAT	choline acetyl transferase
CHF	chronic heart failure
ChTX	charybdotoxin

CIH	chronic intermittent hypoxia
CO	carbon monoxide
Compound C	6-[4-(piperidin-1-yl-ethoxy)-phenyl]-3-pyridin-4-yl-pyrrazolol-[1,5-a]-pyrimidine (AMPK inhibitor)
CSE	cystathione γ lyase
CSN	carotid sinus nerve
D_2	dopamine receptor 2
DA	dopamine
DAB	1, 4-dideoxy-1, 4-imino D-arabinitol hydrochloride (glycogen phosphorylase inhibitor)
DAG	diacylglycerol
DMSO	dimethyl sulphoxide
DNP	2,4-dinitrophenol
ECA	external carotid artery
E_m	membrane potential
ENT	equilibrative adenosine transporter
EPAC	exchange protein activated by cAMP
$FADH_2$	reduced flavin adenine dinucleotide
FBS	foetal bovine serum
FCCP	carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
GE	glucose stimulated neurone
GI	glucose inhibited neurone
GLUT	glucose transporter
GSH	reduced glutathione
GSSG	glutathione disulphide
H_2O_2	hydrogen peroxide
H_2S	hydrogen sulphide
HEK 293	human embryonic kidney 293 cell line
HEX	hexamethonium
HIF	hypoxia inducible factor
hx	hypoxia
HO	haemoxygenase
ICA	internal carotid artery
K_m	Michaelis constant

KO	knock out
LH	lateral hypothalamic region
Lkb1	liver kinase B1
MABP	mean arterial blood pressure
Mb	myoglobin
MO25	mouse protein 25
MSNA	muscle sympathetic nerve activity
NAC	N-acetylcysteine
NBTI	S-(4-nitrobenzyl)-6-thioinosine
nAChR	nicotinic acetylcholine receptor
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
NO_2^-	nitrite
NOS	nitric oxide synthase
NOX	NADPH oxidase
NTS	nucleus tractus solitarius
O_2^-	superoxide anion
OA	occipital artery
P_2	purinergic receptor 2
pH_i	intracellular pH
pH_o	extracellular pH
PC12	pheochromocytoma 12 cell line
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PP2C	protein phosphatase 2C
ROS	reactive oxygen species
R-N-H_2	amine group
R-S-H	thiol group
R-SNO	S-nitrosated thiol group
RT-PCR	reverse transcription polymerase chain reaction
sAC	soluble adenylate cyclase
SCG	superior cervical ganglion

SDB	sleep disordered breathing
SDHD	succinate dehydrogenase anchoring protein
SiRNA	small interfering ribonucleic acid
sLTF	sensory long term facilitation
SNA	sympathetic nerve activity
SOD	superoxide dismutase
STRAD	Ste20-related adaptor
TASK	TWIK-related acid sensitive K^+ channel
TEA	tetraethylammonium
Thr 172	threonine residue on the α subunit of AMPK
tmAC	transmembrane adenylate cyclase
TWIK	two pore domain K^+ channel
TREK	TWIK-related K^+ channel
TH	tyrosine hydroxylase
VChaT	vesicular acetylcholine transporter
V_E	minute ventilation
VO_2	O_2 consumption
WT	wild type

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1. Introduction

1.1 Summative overview

The type I cell in the adult mammalian carotid body (CB) is widely acknowledged to be the key peripheral, systemic chemoreceptor, stimulated by acute hypoxia. CB activation in hypoxia evokes a series of well characterised respiratory and cardiovascular reflex responses and promotes the corrective regulation of arterial O_2 tension and cellular metabolism throughout the whole organism (1). It is becoming more apparent that chronic up-regulation of these reflex pathways, secondary to considerable modification in CB sensory activity, is associated with a number of important clinical conditions or diseases including sleep disordered breathing (SDB), chronic heart failure (CHF) and spontaneous/essential hypertension (2). The identification of selective pharmacological agents capable of reversing the increase in basal CB chemoafferent discharge frequency and CB hypersensitivity to hypoxia in these disease states may prove to be of significant clinical importance for improving patient prognoses.

The development of clinical interventions has, however, been limited due to the fact that the fundamental mechanism underpinning CB activation by hypoxia remains elusive. The essential feature of the O_2 sensor within the type I cell must be its ability to respond to variations in O_2 tension well above those that impact on the metabolism of other cell types. Whilst a considerable number of hypotheses have been proposed to account for this specific behaviour, none are currently universally accepted. Of all the proposed O_2 sensors only the type I cell mitochondria appear to be particularly sensitive to O_2

tensions within the physiological range at which the CB is stimulated (3, 4). Conclusive evidence demonstrating that impairment of mitochondrial function is causative of CB stimulation in hypoxia is yet to be reported. Initial experiments in this thesis therefore aimed to more clearly establish or reject a role for the mitochondria in CB O_2 sensing and provide evidence supporting the hypothesis that impairment of mitochondrial function is a fundamental process in the CB hypoxia stimulus response coupling signalling cascade.

It has been hypothesised that a hypoxia induced reduction in mitochondrial electron transport and ATP synthesis leads directly to an increase in the AMP:ATP ratio and activation of AMP activated protein kinase (AMPK); a protein regarded as a global sensor of cellular energy status (5, 6). Phosphorylation of AMPK by Liver kinase B 1 (Lkb1) is necessary to achieve full activation of AMPK (7). Whilst it is clear that pharmacological activation of AMPK stimulates the CB (5), it is not clear whether activation of the Lkb1-AMPK signalling cascade is the key to activating the CB in hypoxia. Experiments utilising transgenic animals were designed to test this possibility and demonstrate an essential role for Lkb1 in the full expression of O_2 sensitivity in the CB.

In addition to hypoxia, it has been proposed that the CB responds to a number of other blood-borne stimuli including hypercapnia, acidosis and low glucose. The direct sensitivity of the CB to low glucose is still somewhat controversial and a number of clear discrepancies have been reported in the literature. The acute low glucose sensitivity of the intact *in vitro* CB was examined in this study. The results indicate that direct low glucose sensing by the CB is unlikely and responses to prolonged glucose deprivation

only occur secondary to the depletion in glycogen and a reduction in cellular energy status. The potential for artefact in various models of acute glucose sensing is considered along with an indirect sensory role for the CB in systemic glucose homeostasis.

The sensory activity of the CB may be modified by a number of intracellular and extracellular mediators. A comprehensive understanding of the physiological role of these factors and characterisation of the downstream signalling pathways may allow for development of additional therapeutic agents to adjust CB excitability in certain pathologies. A number of endogenous gasotransmitters have been implicated in mediating CB sensitivity to hypoxia including hydrogen sulphide (H_2S), carbon monoxide (CO) and nitric oxide (NO) (8). Using the novel mitochondrial NO donor, nitrite (NO_2^-), the direct impact of NO on modifying mitochondrial activity and CB hypoxic sensitivity was directly investigated in this study. The findings presented suggest that exogenous NO_2^- impairs type I cell mitochondrial electron transport and enhances the CB sensitivity to hypoxia.

The interaction between a host of different neurotransmitters and neuromodulators may also contribute in establishing the overall sensitivity of the CB to physiological or pathological stimuli. Of these, endogenously produced adenosine has often been regarded as an overlooked signalling molecule in the CB (9). Significant quantities of adenosine may be generated in the CB as a consequence of intracellular or extracellular catabolism of ATP (10, 11). Experiments described in this thesis identify an important and novel functional role for extracellular derived adenosine in modulating the CB chemoafferent response to a number of different stimuli.

1.2 Mechanisms of acute oxygen sensing in the mammalian carotid body

1.2.1 Basic anatomy and histology of the carotid body

In mammals the CB is located just above the carotid bifurcation and is closely associated with a number of surrounding structures including the internal carotid artery (ICA), the external carotid artery (ECA), the occipital artery (OA) and the superior cervical ganglion (SCG). Measurements in adult rats have shown that the CB has an oval shape with an average length of approximately 600 µm and a diameter of approximately 200-300 µm (12).

Within a superficial connective tissue capsule that surrounds the CB, there are many small clusters of between 2-12 type I cells, which are associated with a smaller number of glial-like type II cells amidst a dense arrangement of nerve bundles and blood vessels (13). Type I cells are thought to be the specific secretory cells of the CB containing many dense core vesicles, Golgi apparatus and mitochondria (14). The expression of the gap junction subunit connexin 43 in type I cells has been detected (15), supporting the proposal that clusters of type I cells are electrically coupled to function as discrete secretory units.

The CB receives afferent innervation from the carotid sinus nerve (CSN), a branch of the glossopharyngeal nerve. The cell bodies of the sensory chemoafferent fibres are located in the petrosal ganglion and the axonal terminals form synapses with neurones in the nucleus tractus solitarius (NTS) in the medulla oblongata. Electron micrographs of CB slices have demonstrated that the type I cells and the afferent fibres form specific

synaptic connections, consistent with the notion that type I cells have an important role in transmitting sensory information through the CSN into the central nervous system (16).

For its size, the CB has an extremely high blood flow, with a proposed estimate being approximately 2 L.100 g^{-1} tissue.min^{-1} (17). Innervation of the dense vasculature is provided by sympathetic noradrenergic fibres that originate in the superior cervical ganglion (16). Therefore, it appears that there exists a specific mechanism for the acute control of the local CB blood flow and O_2 delivery.

1.2.2 Established hypoxic chemotransduction processes in the mammalian carotid body

A number of specific processes involved in CB hypoxia stimulus response coupling have been characterised to date. These include the attenuation of outward K^+ channel current, type I cell depolarisation, influx of Ca^{2+} through L-type Ca^{2+} channels, type I cell neurosecretion and an increase in the discharge frequency of chemoafferent neurones in the CSN (18).

Type I cell depolarisation

The resting membrane potential (E_m) of the CB type I cell is thought to be approximately -40 mV in the rabbit (19) and between -55 and -70 mV in the rat (20). Reports have demonstrated that exposure of rabbit type I cells to hypoxia evoked a cellular depolarisation that was sustained throughout the length of stimulus duration (19). The magnitude of the depolarisation was approximately +30 mV, thereby raising the type I cell membrane potential to approximately -10 mV. A comparable degree of

depolarisation has also been detected in rat type I cells during hypoxia (21) or anoxia (20, 22).

Inhibition of outward K^+ current

The depolarisation of type I cells in response to hypoxic stimulation is thought to be primarily a consequence of the attenuation of the outward K^+ channel current. A number of studies utilising whole cell voltage clamp techniques have shown that hypoxia significantly depressed the outward K^+ current over a voltage range of -10 to +70 mV (19, 23, 24). Recordings of single K^+ channel activity have revealed a marked reduction in open probability upon exposure to hypoxia, in both cell attached (19) and excised inside out (23) patch clamp configurations.

Full characterisation of the type and contribution of the individual K^+ channels involved in CB O_2 sensing is still to be determined. In rat type I cells it has been demonstrated that measurable whole cell outward K^+ currents were observed at voltages positive to -30 mV (25). This outward K^+ current exhibited Ca^{2+} sensitive and Ca^{2+} insensitive components. Since the K^+ current was significantly attenuated in the presence of charybdotoxin (ChTX), it was proposed that this Ca^{2+} dependent K^+ current was carried specifically through the large conductance Ca^{2+} activated K^+ channel (BK_{Ca}).

In a later study, it was observed that ChTX depolarised the type I cell in a manner that mimicked the effect of hypoxia (22). It was shown that a reduction in outward K^+ current induced by ChTX was not further suppressed by hypoxia and this led authors to conclude that inhibition of the BK_{Ca} channel was necessary for evoking type I cell depolarisation during hypoxia (21).

The role of the BK_{Ca} channel in mediating the initial hypoxia induced cellular depolarisation has subsequently been questioned since it shows little activity at potentials less than -30 mV (the rat type I cell E_m is approximately -70 to -55 mV) and is therefore unlikely to contribute to the E_m. It has also been reported that a number of different inhibitors of K_v and BK_{Ca} channels (tetraethylammonium; TEA, 4-aminopyridine; 4-AP and ChTX) all failed to directly stimulate the type I cell (20). In addition, direct addition of ChTX to the intact *in vitro* CB does not seem capable of directly enhancing chemoafferent activity (26). For these reasons, a functional role for the BK_{Ca} channel in physiological O_2 sensing in the CB remains debatable.

Emerging evidence supports the proposal that modified activity of specific members of the two-pore-domain K^+ channel sub-family regulates type I cell depolarisation in hypoxia. A background K^+ current in the type I cell was first identified by Buckler in 1997 (20). This current was measurable over a voltage range of -90 to -30 mV and was impaired by anoxia. Recordings of single channel activity made over a voltage range of -70 to -60 mV detected single K^+ channel activity in normoxia that was significantly diminished by hypoxia (27). A number of further observations suggested that this background O_2 sensitive current was carried through a TASK-like (TWIK-related acid sensitive K^+) channel. Firstly, the current was attenuated by known inhibitors of TASK channels; Ba^{2+}, Zn^{2+} and quinidine. Secondly, the current was sensitive to acid. Thirdly, the single channel conductance was consistent with values reported for TASK-1. And finally, in situ hybridisation positively identified TASK-1 mRNA in dissociated type I cells. More recently, another member of the two-pore-domain K^+ channel sub-family, TREK-1 (TWIK related K^+ channel 1) has been identified in type I cells (28) and it has been

shown that the TREK-1 conductance was almost completely abolished by hypoxia (29). These data suggest that deactivation of the TASK-like and TREK-1 channels may both be significant in eliciting type I cell depolarisation in hypoxia.

Ca^{2+} influx

It is now recognised that hypoxia induced type I cell depolarisation leads to a rapid rise in $[Ca^{2+}]_i$ and this in turn stimulates the release of stored excitatory and inhibitory neurotransmitters (30-33). Measurements of type I cell Ca^{2+} fluorescence indicate that the degree of $[Ca^{2+}]_i$ elevation is highly dependent on the level of hypoxic stimulus intensity (30). The increase in $[Ca^{2+}]_i$ is completely abolished in Ca^{2+} free media (31). Furthermore, the anoxia induced augmentation of type I cell $[Ca^{2+}]_i$ coincides with cellular depolarisation and is almost completely ablated by Ni^{2+} (a non-specific Ca^{2+} channel antagonist) and by nicardipine (a specific L-type Ca^{2+} channel blocker) (31). Collectively these data indicate that the vast majority of the elevation in $[Ca^{2+}]_i$ in hypoxia is dependent on Ca^{2+} influx through voltage sensitive L-type Ca^{2+} channels.

Neurosecretion

It is well documented that hypoxia stimulates the release of a number of different stored neurotransmitters from the type I cells. The two best characterised excitatory neurotransmitters are ATP (34) and ACh (35, 36). The release of ATP in hypoxia appears to be critically dependent on the preservation of L-type Ca^{2+} channel activity, indicative of the ATP secretion being secondary to the initial rise in $[Ca^{2+}]_i$ (34). Emerging evidence also supports the idea that synaptic adenosine concentrations increase in hypoxia and have an important excitatory neuromodulatory role in regulating the chemoafferent response to hypoxia (10, 11). Whether or not the elevation in

adenosine concentration is a consequence of type I cell adenosine release or occurs secondary to extracellular ATP catabolism is unresolved. A number of studies have also detected a rise in CB dopamine (DA) secretion in response to hypoxic stimulation (33, 37). As with ATP, the release of DA is known to be dependent on Ca^{2+} influx through L-type Ca^{2+} channels (38). In contrast to the excitatory neurotransmitters, DA seems to exert an autoinhibitory action on the type I cell sensory response to hypoxia, specifically by attenuating inward Ca^{2+} currents (39). A more detailed consideration of the role of neurotransmitters in CB hypoxia stimulus excitation coupling is presented in Section 1.8.

Stimulation of CSN chemoafferent fibres

The overall functional effect of hypoxia induced type I cell depolarisation, Ca^{2+} influx and neurosecretion is the stimulation of the chemoafferent fibres in the CSN and subsequent transmission of this neural signal into the CNS. This initiates a series of well characterised reflex changes in cardiovascular and respiratory function that allow for corrective regulation of arterial O_2 tension and the maintenance of cellular metabolism throughout the whole organism (1).

The hypoxia evoked increase in single fibre chemoafferent firing frequency has been described in multiple studies both *in vivo* (40, 41) and *in vitro* (42-46). Recordings of single fibre chemoafferent activity made *in vivo*, have identified a normoxic frequency of approximately 0.2 to 2.3 Hz in the cat (47) and 0.25 to 1.5 Hz in the rat (41). In severe hyperoxia (up to 600 mmHg P_aO_2) the single fibre frequency is depressed, but not completely abolished (40), consistent with a degree of tonic O_2 independent neurotransmitter release or spontaneous neuronal activity.

The precise arterial O_2 tension at which the discharge frequency begins to increase is variable but it appears to lie somewhere between a P_aO_2 of 60 and 100 mmHg (41, 47). It is recognised that as the P_aO_2 continues to decrease, the single fibre discharge frequency increases exponentially and peaks *in vivo* between 9 and 25 Hz (41, 47). This hypoxia stimulated exponential rise in chemoafferent activity has also been demonstrated in multiple *in vitro* intact CB preparations (42, 45, 46). The hypoxic response curves generated from the superfused *in vitro* CB preparations seem to be relatively 'right shifted' compared to those obtained *in vivo*. This discrepancy is most likely a consequence of the diffusion limitations present across the whole organ (48). If the hypoxic stimulus is sustained for more than 5 to 10 minutes the peak discharge frequency is not maintained but gradually begins to decline (42).

Summary

All of the sensory transduction processes described above (from the attenuation of outward K^+ channel current, to the exponential elevation in chemoafferent discharge frequency) are well established and are summarised in Figure 1.1. However, characterisation of a detailed interaction between O_2 and a CB specific O_2 binding molecule or sensor, that couples directly to K^+ channel deactivation, remains elusive. A number of hypotheses have been proposed and some are described in the ensuing sections. One of these hypotheses, the 'mitochondrial' hypothesis, is examined directly in this thesis. Modulation of the sensory chemotransduction processes by a number of chemical mediators may also impact on the overall function of the CB. Some of these potential mediators are introduced later in this chapter and are subsequently investigated directly in this thesis.

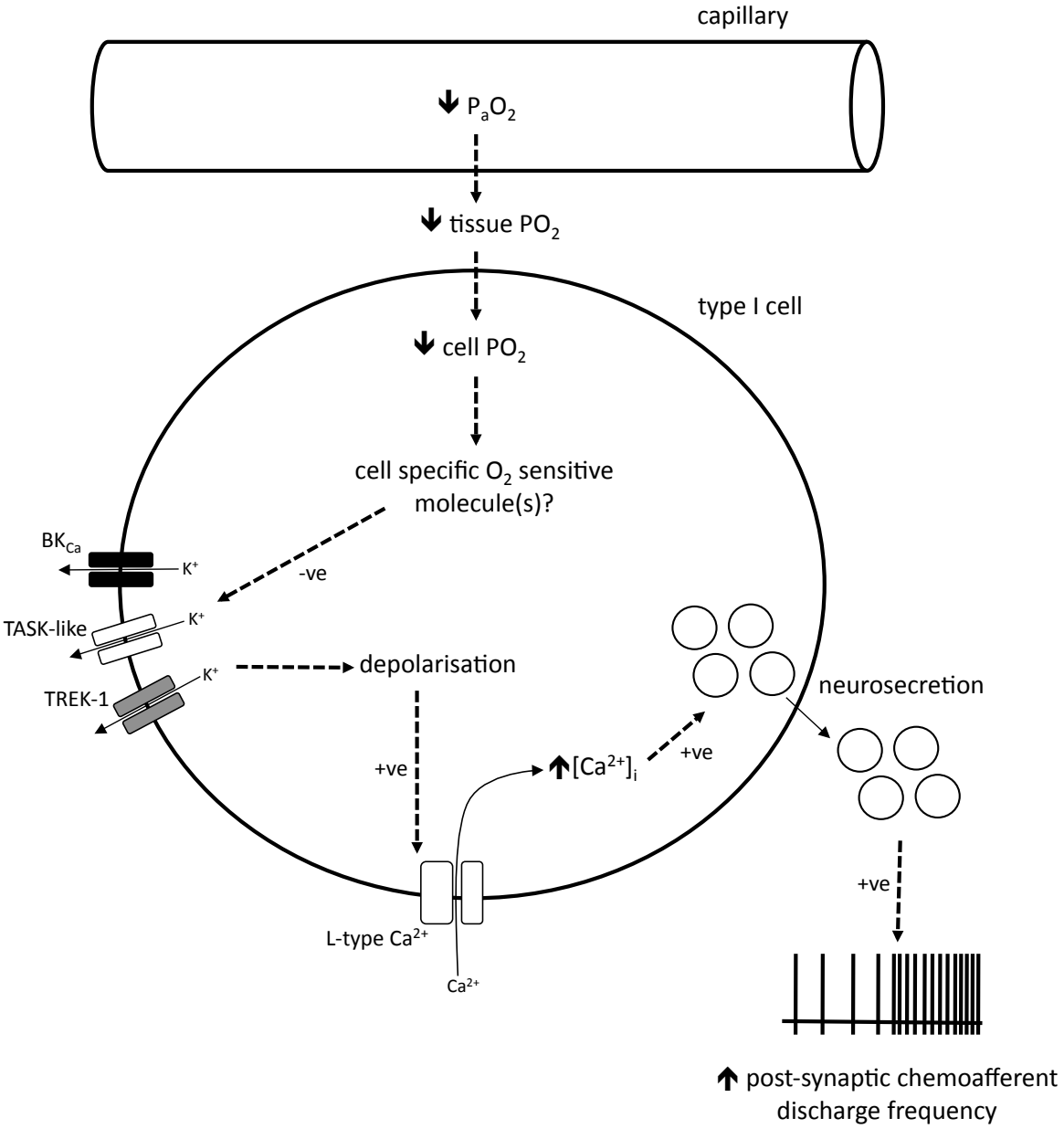


Figure 1.1 Hypoxic chemotransduction processes in the carotid body.
The carotid body is stimulated in response to reductions in arterial and tissue O_2 tensions at levels well above those that impact on the metabolism of other cell types. Three K^+ channels have been implicated in carotid body O_2 sensing including BK_{Ca}, TASK and TREK-1. Attenuation of these channels in hypoxia reduces the outward K^+ current and causes cellular depolarisation. This in turn activates voltage sensitive L-type Ca^{2+} channels leading to Ca^{2+} influx and a rise in type I cell $[Ca^{2+}]_i$. The elevation in $[Ca^{2+}]_i$ promotes the release of stored neurotransmitters that are responsible for increasing the action potential generation in the adjacent chemoafferent neurone. Precise characterisation of a type I cell specific O_2 sensitive molecule (hypoxic sensor) that couples directly to inhibition of the K^+ channels in hypoxia remains elusive.

1.3 The mitochondrial hypothesis

1.3.1 Chemoexcitation evoked through mitochondrial inhibition and hypoxia share a number of similar chemotransduction processes

In mammalian cells, O_2 is the terminal electron acceptor in the mitochondrial respiratory chain. Continuous binding and reduction of O_2 in the Cu_B/haem a_3 binuclear centre of complex IV drives mitochondrial electron transport and promotes activation of the mitochondrial ATP synthase. The 'mitochondrial' hypothesis for chemoreception proposes that CB excitation induced by hypoxia is initiated by a reduction in O_2 dependent mitochondrial energy respiration.

It is well established that mitochondrial poisons cause intense CB chemostimulation. This was originally identified in an early report demonstrating that injection of cyanide into the carotid sinus region immediately evoked an increase in minute ventilation that was dependent on an intact CSN (49). It is now recognised that the elevation in chemoafferent activity induced by saturating doses of mitochondrial poisons is similar in magnitude to that evoked by severe hypoxia (50-52). Mitochondrial inhibitors or uncoupling agents also enhance ^{3}H-DA neurotransmitter release from the *in vitro* CB, indicating that the excitatory actions of these compounds is mediated through the actual type I cell and not the afferent nerve ending (52, 53).

It is widely accepted that mitochondrial poisons are able to induce rapid increases in type I cell $[Ca^{2+}]_i$. The scale and immediacy of the $[Ca^{2+}]_i$ elevation in response to a range of different mitochondrial inhibitors or uncouplers closely resembles the action of hypoxia (30, 54, 55). Furthermore, the rise in $[Ca^{2+}]_i$ is highly dependent on extracellular

Ca^{2+} and almost completely reliant on cellular depolarisation (54). This implies that the vast majority of the rise in $[Ca^{2+}]_i$ induced by mitochondrial poisons, as with hypoxia, is a consequence of Ca^{2+} influx through voltage gated Ca^{2+} channels. However, a small persistent elevation in $[Ca^{2+}]_i$ has been observed even in the absence of extracellular Ca^{2+} or when type I cells were voltage clamped (54, 56). A definitive mechanism underpinning this residual rise in Ca^{2+} is equivocal but may involve the release of Ca^{2+} from an intra-mitochondrial store (56) or Ca^{2+} entry following acid induced reversal of the Na^+/Ca^{2+} exchanger (53).

A number of different K^+ channels have been implicated in CB hypoxia sensing including BK_{Ca} (21, 22, 57), TASK (27) and TREK-1 (29). As with hypoxia, rotenone, cyanide and FCCP all attenuate the background TASK-like current in isolated CB type I cells (54). It has also been reported that complex IV inhibition with sodium azide leads to the depression of TASK and TREK-1 currents (29). Type I cell depolarisation evoked by a run-down in mitochondrial energy metabolism is therefore likely to involve at least two K^+ channels that are associated with CB O_2 sensing.

1.3.2 The mitochondria in the carotid body type I cells appear to have a uniquely low affinity for O_2

A fundamental feature of the CB type I cell is its ability to respond to hypoxic stimuli at PO_2 levels well above those that evoke changes in the metabolism of other cell types. For the mitochondrial hypothesis to be validated the mitochondria in the type I cells would have to show some degree of highly specialised low affinity binding for O_2 that made mitochondrial electron flux significantly more susceptible to changes in PO_2.

The K_m of the cytochrome a_3 for O_2 in the mammalian mitochondrial complex IV is reported to be < 1 mmHg in isolated mitochondria and between 1 and 5 mmHg in dissociated cells and tissue preparations, with no significant differences existing between most cell varieties (58, 59). Within the CB, it has been established that there exists a cytochrome a_3 with an unusually low binding affinity for O_2, with a significant degree of it being reduced at PO_2 tensions as high as 40 to 80 mmHg in the perfused organ preparation (3, 60, 61).

Consistent with these findings, later studies performed by Biscoe and Duchen showed that in isolated type I cell clusters, mitochondrial function, measured by NADH autofluorescence, started to be impaired at a PO_2 value of approximately 40 mmHg (4). Construction of PO_2-NADH response curves revealed that the mitochondrial function in the type I cell was much more susceptible to a fall in PO_2 compared to sensory neurones, as evidenced by the curves generated from type I cells being significantly 'right shifted'. Mitochondrial depolarisation, measured by rhodamine 123 fluorescence, was also detected at significantly higher O_2 tensions in type I cells (56). In view of these observations, it does appear that type I cell mitochondria have a highly specialised low affinity for O_2 and that mitochondrial energy metabolism may begin to be inhibited at remarkably high O_2 tensions.

1.3.3 Interaction between mitochondrial inhibitors and hypoxia

The degree of stimulation elicited by mitochondrial poisons is similar to that evoked by severe hypoxia/anoxia in terms of the extent of mitochondrial depolarisation, the rise in NADH autofluorescence, elevation of $[Ca^{2+}]_i$ and augmentation of chemoafferent discharge frequency (4, 52, 54, 56, 62). Wyatt and colleagues showed that the

attenuation of TASK-like current by high doses of rotenone, cyanide and FCCP was not further depressed by hypoxia (54). This perhaps suggests that all of these stimuli act through a common sensory pathway. However, it could be argued that the suppression of the TASK-like current evoked by the mitochondrial poisons was not further decreased by hypoxia because the maximal decline in TASK-like current had already been attained.

In a different study, using the CB slice preparation, application of a range of mitochondrial inhibitors increased the basal level of DA secretion but did not prevent additional responses to hypoxia (63). The same group demonstrated that CB slices isolated from mice partly deficient in the succinate dehydrogenase anchoring protein (SDHD) had a preserved secretory response to hypoxia (64). In view of these results it was concluded that impairment of mitochondrial electron transport was not necessary for coupling hypoxia with CB excitation. However, the authors did not examine a potential change in CB hypoxic sensitivity in the SDHD heterozygous KO mice or following inhibition of the mitochondria using pharmacological agents. This may have been crucial for their conclusions to be validated, given that mitochondrial activity was likely to have only been partially attenuated.

For the impairment of mitochondrial function to be contributory to CB hypoxic chemoexcitation, when sub-saturating concentrations of mitochondrial inhibitors are applied, it would be predicted that the threshold required for hypoxic response initiation would be modified. Mild mitochondrial inhibitors would be expected to sensitise the CB to hypoxia. Due to the exponential shape of the CB hypoxic response curves, an interaction of this nature would produce multiplicative rather than additive responses. Up

to now, no investigation has examined continuous graded responses to hypoxia in the presence of mild mitochondrial inhibitors. A better knowledge of the interaction between these two stimuli may be important to more clearly establish or rule out a role for mitochondrial energy metabolism in the CB hypoxic chemotransduction process and for this reason it is examined directly in this thesis.

1.3.4 Is hypoxia coupled to a decrease in $[ATP]_i$?

If the rate of mitochondrial electron transport is attenuated during hypoxia, it may be reasonable to hypothesise that this would be coupled to a decrease in mitochondrial ATP generation and a reduction in the cellular energy status. As yet, no evidence has been published reporting the precise type I cell $[ATP]_i$, either in normoxic conditions or during hypoxia. In other cell types including skeletal muscle cells and hepatocytes, very severe hypoxia (< 1% O_2) reduces the $[ATP]_i$ consistent with a fall in the rate of oxidative phosphorylation (65, 66). In acutely O_2 sensitive pulmonary artery smooth muscle cells, hypoxia enhanced $[Ca^{2+}]_i$, and this was associated with an increase in the AMP:ATP ratio, indicative of a concurrent depletion in cellular energy status (5).

For CB tissue, measurements of whole organ ATP content have been made to reflect potential alterations in type I cell $[ATP]_i$. Studies have reported that hypoxia and metabolic inhibitors, applied at an intensity sufficient to evoke intense CSN stimulation and ^{3}H-CA release, reduced the whole CB ATP content (67, 68). It has also been shown that exposure of intact CBs to hypoxia, cyanide or antimycin A caused a significant depletion in whole organ ATP and an increase in AMP content (69).

In direct contrast to the above studies, it has been observed that the ATP content of the whole CB was not significantly diminished following whole animal exposure to severe hypoxia (5% O_2 in N_2) (70). Another investigation reported that two different mitochondrial uncoupling agents, DNP and CCCP, both increased the CSN discharge frequency without reducing ATP content (52). At present, there seems to be no clear correlation between metabolic or hypoxic induced stimulation and a variation in whole CB ATP content.

In all of the above studies, the measured ATP content will have had contributions from type I cells, type II cells, neurones, blood vessels and interstitial fluid. In view of this, it is hard to justify that any alteration in whole organ ATP content accurately reflects an equivalent adjustment in type I cell $[ATP]_i$. Conclusions are further complicated by the fact that ATP is an established neurotransmitter that is stored in type I cells and is released in hypoxia (71, 72). Therefore, whether or not type I cell $[ATP]_i$ or the AMP:ATP ratio are modified directly by hypoxia remains to be resolved.

1.3.5 Is chemoexcitation evoked in hypoxia dependent on mitochondrial reactive oxygen species generation?

Generation of reactive oxygen species (ROS) has been implicated in a number of important physiological processes in mammals including the control of the systemic vascular tone, pulmonary artery vasoconstriction and production of erythropoietin (reviewed in detail in (73)). In the mitochondria, it has been estimated that 1 to 2% of O_2 is not completely converted to H_2O but instead is reduced by single electron addition to form the highly reactive superoxide anion O_2^- (74). O_2^- conversion to hydrogen peroxide (H_2O_2) is catalysed by specific superoxide dismutases (SOD) within the mitochondria

(MnSOD) or the cytosol (Cu/ZnSOD) (75). Catalase activity leads to thermodynamically favourable decomposition of H_2O_2 to form of H_2O and O_2. In the presence of certain transition metals such as Fe^{2+}, H_2O_2 may be cleaved to produce the highly unstable hydroxyl radical ($OH^{\bullet}$); the third major ROS (76).

The formation of O_2^- is thought to be dependent on the concentration of O_2 as defined by the reaction;

$$d[O_2^-]/dt \propto [O_2].[R^{\bullet}],$$

where t is time and $R^{\bullet}$ is the concentration of available reducing equivalents (77). The majority of cellular ROS is generated from initial formation of O_2^- in the mitochondria (78), primarily a consequence of electron leakage at mitochondrial complexes I and III. It has been demonstrated that in isolated mitochondria, formation of O_2^- was favoured in hyperoxic conditions (79).

Strong evidence also supports the hypothesis that the upregulation of mitochondrial ROS formation in hypoxia is essential for pulmonary artery vasoconstriction (80). The mechanism underpinning the elevation in $[O_2^-]_i$ in hypoxia is still not understood and appears to be contradictory to the idea that the rate of O_2^- production is dependent on mass action as described by the above reaction. It has been suggested that in hypoxia, a run-down in the rate of electron flux increases the electron ‘dwell time’ on certain complexes of the mitochondrial respiratory chain and augments the number of reducing equivalents (81). This in turn would promote mitochondrial O_2 reduction and enhance O_2^- production. A similar mechanism is thought to account for the increase in complex III

ROS generation observed in the presence of mitochondrial inhibitors antimycin A and cyanide (81).

It has been observed that ROS production was enhanced by hypoxia in both the mouse (82) and rat (83) CB type I cells. Despite this, ROS formation is not thought of as the fundamental process that links hypoxia with type I cell activation. Very high exogenous H_2O_2 administration (up to 632 mM) has been reported to activate rather than inhibit TASK channel activity in type I cells, an effect that would tend to cause hyperpolarisation rather than depolarisation (84). In the same study, H_2O_2 had no impact on the open probability of the O_2 sensitive TREK-1 and BK_{Ca} channels. Changes in type I cell $[Ca^{2+}]_i$ also seem to be insensitive to H_2O_2 when applied under normoxic or hypoxic conditions (54).

In another study, a series of oxidants and inhibitors of ROS disposing enzymes were used to monitor the potential effect of redox homeostasis on CB activation by hypoxia. All drugs were reported to decrease the reduced glutathione (GSH) to oxidised glutathione (glutathione disulphide; GSSG) ratio in isolated diaphragm, consistent with a switch to a relatively oxidised intracellular redox environment (85). A number of these oxidising agents did augment CB ^{3}H-CA release, but others were found to have no effect. This apparent lack of correlation between redox status and CB stimulation led authors to conclude that hypoxic chemotransduction was not dependent on ROS generation. This was further supported by observations showing that *in vivo* administration of buthionine sulphoximine (an inhibitor of GSH synthesis) failed to significantly alter the ventilatory response to hypoxia.

The same group also revealed that N-acetylcysteine (NAC; a potent antioxidant) reversed the change in redox status evoked by a number of mitochondrial poisons but had no impact on the CB secretory response induced by these same agents (86). Finally, NAC applied to the isolated calf CB, despite augmenting the GSH/GSSG ratio, did not modify the level of ^{3}H-CA release in normoxia or hypoxia (87).

Despite a variation in the type I cell redox status appearing not to be directly implicated in acute CB O_2 sensing, a chronic upregulation in local ROS production has been suggested to account for the increase in basal chemoafferent activity and heightened CB hypoxic sensitivity in a number of different pathologies (discussed in Section 1.9).

1.4 Linking cellular metabolic stress to type I cell activation: a role for the Lkb1-AMPK pathway

1.4.1 Basic properties of AMPK and Lkb1

It has been proposed that stimulation of AMPK, secondary to a hypoxia induced change in cellular energy status, is necessary to activate the CB in hypoxia (5, 88). AMPK is a heterotrimeric protein complex composed of the catalytic α and regulatory β and γ subunits (89), and for each subunit multiple different isoforms are expressed. AMPK is often referred to as the global sensor of cellular energy status because of its ability to acutely respond to changes in the AMP:ATP ratio (6).

Regulation of AMPK activity is complex and highly integrated. It is now accepted that phosphorylation of the specific threonine residue (Thr 172) in the N-terminal kinase domain on the catalytic α subunit by upstream kinases is required to achieve full

activation (90). In addition, the regulatory γ subunit contains two pairs of adenosine nucleotide binding motifs termed ‘Bateman’ or cystathione β synthase (CBS) domains (labelled sites 1–4) (91, 92). Observations of the partial crystal structure of AMPK identified that only 3 of the 4 ‘Bateman’ domains could be occupied by adenosine nucleotides (with site 2 adopting a permanently ‘closed’ conformation) and one of these domains, site 4, was permanently bound to AMP (93). Reversible and competitive binding of ATP, ADP and AMP is only considered to take place at sites 1 and 3 and variations in the occupancy of these sites by the different nucleotides is thought to account for the overall degree of AMPK activation (94).

AMP is the only adenosine nucleotide that allosterically activates AMPK and complete saturation of the exposed CBS motifs produces a modest 1.5–3 fold elevation in activity (95, 96). However, AMP also enhances α subunit phosphorylation (95) and restricts the rate of α subunit dephosphorylation by protein phosphatase 2C (PP2C) (97). In combination, the overall impact of AMP is to elevate AMPK activity by approximately 1000 fold (96). ADP is another adenosine nucleotide capable of binding to and activating AMPK (94). It is thought that this is achieved not through direct allosteric activation but by attenuating PP2C mediated Thr 172 dephosphorylation.

The ability for ATP to bind to the available CBS domains on α subunit of AMPK is well characterised. ATP effectively deactivates AMPK by competing with AMP and ADP for the occupancy of these two ‘free’ CBS domains (94, 95). The overall regulation of AMPK phosphorylation, dephosphorylation and activation is therefore highly dependent on the relative intracellular concentration of ATP, ADP and AMP.

For a number of years the identity of the upstream AMPK kinase remained elusive. In 2003, Woods and colleagues detected a 45 kDa fragment from the isolated and purified rat liver AMPK kinase that reacted positively with anti Lkb1 (Liver kinase B 1) antibodies (7). The authors went on to demonstrate that purified Lkb1 activated AMPK by targeted phosphorylation of the Thr 172 residue in the activation loop on the catalytic α subunit. Furthermore, pharmacological activation of AMPK was prevented in cells expressing a mutant deactivated form of Lkb1. These were the first observations implying that Lkb1 is the essential upstream kinase required for complete phosphorylation and activation of AMPK.

It is now established that Lkb1 exists as part of a globular 1:1:1 heterotrimeric complex in combination with two other proteins; STRAD (Ste20-related adaptor) and MO25 (Mouse protein 25) (98, 99). The whole Lkb1-STRAD-MO25 complex phosphorylates the Thr 172 residue in the activation loop on the catalytic α subunit of AMPK (98). The degree of AMPK activation is closely associated with the level of Lkb1 activity. Full activation of AMPK is only achieved in the presence of Lkb1 and both STRAD and MO25 peptides. Direct interactions between STRAD/MO25 and Lkb1 cause allosteric activation of Lkb1 independent of phosphorylation (99). For this reason STRAD and MO25 are regarded as important regulators of Lkb1 function. The regulation of AMPK activity by Lkb1 and adenosine nucleotides is illustrated in Figure 1.2.

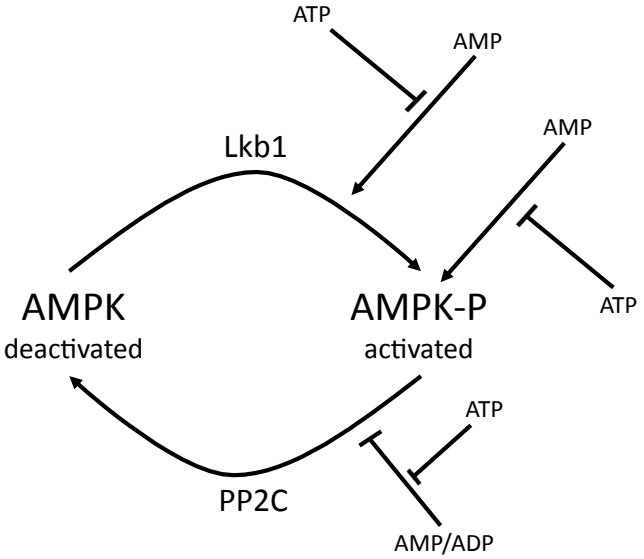


Figure 1.2 Regulation of AMPK phosphorylation status by Lkb1 and adenosine nucleotides. Lkb1 is the key upstream kinase that phosphorylates AMPK and is necessary to achieve complete enzyme activation. AMP binding to the CBS domains on the regulatory γ subunit increases AMPK activation by 1) direct allosteric activation 2) enhancing Lkb1 phosphorylation and 3) inhibiting AMPK dephosphorylation by PP2C. ADP binding at the available CBS domains also restricts AMPK dephosphorylation. ATP competes with AMP and ADP for binding at the CBS domains on the γ subunit and limits AMPK phosphorylation and activation. The phosphorylation status of AMPK is therefore dependent on the AMP:ATP and ADP:ATP ratios. Adapted from Hardie et al. 1999 (6).

1.4.2 Physiological and pharmacological activation of AMPK

A rise in the ADP:ATP ratio is a consequence of the rate of ATP generation being less than the rate of ATP hydrolysis. The rise in $[ADP]_i$ activates adenylate kinase and drives the reaction; $2ADP \Leftrightarrow AMP + ATP$, that acts to partially restore the $[ATP]_i$ (100). As a consequence of the adenylate kinase reaction it has been estimated that the AMP:ATP ratio varies as the square of the ADP:ATP ratio (100).

AMPK can be activated by a number of different physiological or pharmacological stimuli, the majority of which are associated with an induction of metabolic stress. In HEK293 cells over expressing the γ2 subunit of AMPK, it was observed that a number of metabolic poisons including 2-deoxyglucose, oligomycin, DNP and metformin, all activated AMPK and this was coupled with a rise in the ADP:ATP ratio and, in most cases, a fall in O_2 consumption (101). AMPK is also activated in skeletal muscle following periods of stimulated contraction (102). In cell lines derived from pancreatic β-cells, a graded reduction in the superfusate glucose concentration from 10 to 0 mM was shown to evoke a concomitant graded elevation in AMPK activity and a rise in both the AMP:ATP and ADP:ATP ratios (103).

Importantly, hypoxia is acknowledged as a key physiological stimulus that can activate AMPK. For example, it has been identified that the Thr 172 phosphorylation status in cardiac AMPK was significantly enhanced following a 10 minute period of regional cardiac ischaemia *in vivo* (104). In acutely O_2 sensitive smooth muscle cells, hypoxia augmented the level of α subunit phosphorylation and increased the level of acetyl CoA carboxylase phosphorylation (a downstream substrate of AMPK), indicative of an up

regulation in AMPK activation (5). This was associated with an elevation in the AMP:ATP and ADP:ATP ratios. It was also demonstrated that AICAR, a compound that activates AMPK via intracellular generation of ZMP (an AMP analogue), augmented Ca^{2+} influx into smooth muscle cells and evoked vasoconstriction of isolated pulmonary arterioles *in vitro*. These findings described by Evans et al. were the first to reveal that activation of AMPK by AICAR mimicked the physiological action of hypoxia in an O_2 sensitive tissue.

The importance of Lkb1 in regulating AMPK activity when exposed to physiological or pharmacological stimulation is becoming more apparent. For example, it has been reported that Lkb1 deficient murine embryonic fibroblasts, upon exposure to pharmacological AMPK activators, had reduced levels of α subunit Thr 172 phosphorylation and acetyl CoA carboxylase phosphorylation compared with wild type controls (105). Restoration of AMPK phosphorylation and activity was achieved by re-expression of an active form of Lkb1, implying that Lkb1 was essential for AMPK activation in response to these pharmacological stimuli. In mouse heart tissue deficient in Lkb1, AMPK (α2) activity was not enhanced following ischaemia or anoxia (106), emphasising a critical role for Lkb1 in mediating AMPK activation by hypoxia.

1.4.3 Carotid body stimulation by hypoxia; a role for AMPK

Recent evidence suggests that AMPK may be an important component of the signalling cascade that couples hypoxic sensing with CB type I cell excitation. It has been confirmed that the α1 subunit of AMPK is present in the rat type I cells and is co-localised with the BK_{Ca} channel at the plasma membrane (5, 88). A strong degree of mRNA expression encoding the α1 subunit of AMPK has also been detected in CBs

isolated from human patients (107). Functionally, AMPK activation with AICAR mimics the effect of hypoxia by eliciting type I cell Ca^{2+} influx and raising CSN discharge frequency (5). Inhibitory targeting of AMPK with Compound C diminishes the rise in type I cell $[Ca^{2+}]_i$ in response to hypoxia and attenuates the increase in CSN single fibre discharge frequency by approximately 50% (88). Finally, mice deficient in the $\alpha 2$ subunit of AMPK appear to have an impaired hypoxic ventilatory response (108).

In the type I cell, multiple targets of AMPK have been proposed. Wyatt and colleagues observed that exposure of type I cells to AICAR caused a reduction in the BK_{Ca} current (88). This effect was dependent on direct channel phosphorylation and was abolished by AMPK deactivation with Compound C. The same study demonstrated that AICAR attenuated the background TASK-like current, although a specific phosphorylation site was not elucidated. A more recent article has shown that AICAR depressed both the TREK-1 and TREK-2 conductances and this was associated with selective serine residue phosphorylation within the C-terminal domains (29). These data indicate that AMPK phosphorylates a collection of K^+ channels in the type I cell, all of which are closely related to CB O_2 sensing.

1.5 Gasotransmitters in the carotid body; alternative hypotheses for acute hypoxia sensing in the carotid body

The hypothesised mechanisms coupling low O_2 tensions with type I cell stimulation are not only confined to a depletion in mitochondrial electron transport and changes in cellular energy status. Three biologically active gases, carbon monoxide (CO), hydrogen

sulphide (H_2S) and nitric oxide (NO) have all been identified as effectors of the CB response to hypoxia (reviewed in (8)). If high enough concentrations of one or all of these gases are generated under hypoxic conditions then they may act to directly mediate the CB response to hypoxia. The evidence for a physiological role of these gases in CB hypoxia stimulus response coupling is considered in this section.

1.5.1 A role for carbon monoxide in mediating the carotid body response to hypoxia

The impact of CO on CB activity is variable and appears to be directly dependent on the overall $CO:O_2$ ratio. A very high relative proportion of CO compared to O_2 causes intense chemoexcitation (109, 110). Superfusate concentrations of CO (> 300 mmHg) rapidly increase type I cell $[Ca^{2+}]_i$ (111) and enhance the chemoafferent discharge frequency (109, 110). These effects are abolished by antagonism of L-type Ca^{2+} channels, suggesting that the response to CO is secondary to Ca^{2+} influx. CO also suppresses the type I cell resting membrane conductance and evokes cellular depolarisation. Thus, responses to high [CO] are comparable with those seen in hypoxia.

A number of investigations have reported that the CO induced augmentation in chemoafferent activity was reversed by white light (109, 110, 112). The earliest of these demonstrated that the depression in discharge frequency was critically dependent on the wavelength of the illuminating light, being maximal at approximately 430 nm (110). This photosensitivity was consistent with formation of CO-haem complexes. Detailed analysis of the photochemical action spectra revealed that the excitatory effects of CO were probably mediated through binding to cytochrome a_3 in the mitochondria. In view of

these observations, it was concluded that the chemoexcitation induced by [CO] was most likely to be a consequence of the depletion in mitochondrial electron transport.

In biological systems the formation of physiologically active CO is dependent on the catalytic activity of haemoxygenase (HO) (113). CO is produced through the enzymatic degradation of haem in the presence of sufficient O_2 and a number of key co-factors including NADPH and cytochrome P_{450}. Biliverdin is released as a by-product of this reaction (113). Two isoforms of HO have been characterised, the inducible isoform HO-1, detected in liver and spleen where the turnover rate of haem is high, and the constitutively expressed isoform HO-2, predominately present in brain and other neuronal tissue (reviewed in (114)).

It has been proposed that physiological concentrations of CO derived from HO-2 may directly modulate the CB response to hypoxia, in a manner that is independent of an alteration in mitochondrial function (8, 115). Immunocytochemical analysis has revealed that HO-2 is expressed positively in type I cells (116). Pharmacological inhibition of HO-2 elevates type I cell $[Ca^{2+}]_i$ (117) and enhances chemoafferent activity (116), with the latter being reversed by application of exogenous CO. Inhalation of 0.25–2% CO attenuates the hypoxic ventilatory response in the rat (118) and infusion of tin protoporphyrin (another HO-2 inhibitor), augments phrenic nerve activity in 10% O_2, indicative of a sensitised reflex response to hypoxia (114).

Mechanistically, it has been suggested that CO impacts on type I cell excitability by directly interacting with the BK_{Ca} channel (119, 120). Analysis of whole cell patch clamp recordings revealed that the depression of the outward K^+ current induced by hypoxia

was restored by addition of CO (119). A reduction in single K^+ channel open probability in hypoxia was reversed by co-administration of exogenous CO. In HEK293 cells transfected with BK_{Ca}, immunocytochemical analysis was used to demonstrate co-localisation of BK_{Ca} and HO-2 (120). In the same study, knock down of HO-2 expression by siRNA ablated the single K^+ channel activity in normoxia and abolished the channel hypoxic sensitivity. Exogenous application of a CO donor increased the K^+ channel open probability by more than 15 fold. A similar effect was observed upon addition of NADPH and haem, both predicted to elevate HO-2 activity. In a follow up investigation, the CO sensitivity of BK_{Ca} channel was eradicated following complete C-terminal tail substitution (121), implying that CO regulated BK_{Ca} channel function through a specific binding interaction within the C-terminal (Ca^{2+} binding) domain.

As a result of these data, CO has been now been proposed as an important signalling molecule involved in hypoxia stimulus response coupling within the CB (115). It is suggested that, in normoxia, CO generated from HO-2 and haem, increases the open probability of BK_{Ca}, thereby amplifying the constitutive outward K^+ current and preventing type I cell stimulation. In hypoxia, catalytic generation of CO is diminished and the absence of local CO acts to depress BK_{Ca} channel activation and reduces the K^+ current. This evokes type I cell depolarisation, Ca^{2+} influx, neurosecretion and chemoafferent excitation (115).

However, results taken from studies that evaluated the impact of genetic knock down of HO-2 on O_2 sensing indicate that a role for HO-2 derived CO in CB hypoxic signal transduction may not be as definitive as first thought. In HO-2 null mice, minute ventilation seems to be unaffected in normoxia and this is contradictory with the idea

that BK_{Ca} is constitutively active (122). In hypoxia, the overall elevation in minute ventilation is reduced in these mice, but a strong component of the response (approximately 66%) is preserved. Perhaps even more surprising, examination of CB function in HO-2 deficient mice has revealed the conservation of a strong neurotransmitter secretory response to hypoxia coupled with the absence of alteration in hypoxic sensitivity (123). The authors of this study concluded that HO-2 and CO were not required for CB activation by hypoxia. The reason for these apparent contradictory results obtained from genetic and pharmacological studies is yet to be resolved and the physiological action of CO in the CB is still being investigated.

1.5.2 Hydrogen sulphide in carotid body oxygen sensing

Emerging evidence supports the hypothesis that endogenously produced H_2S is a critical signalling molecule in the mammalian CB. H_2S is primarily generated from serine and cysteine amino acid metabolism through a series of reactions that are dependent on the enzymatic activity of cystathione β synthase (CBS) and cystathione γ lyase (CSE) (124, 125). The relative importance of these enzymes in generating H_2S seems to be tissue specific, with CBS accounting for the vast majority of H_2S production in the brain and kidney, whereas in the liver, H_2S is synthesised predominantly via CSE (124, 126).

It is established that H_2S formed from exogenous NaHS, causes a marked degree of respiratory stimulation in a number of different mammalian species including the mouse (127), rat (128) and the sheep (129). Observations from *in vitro* CB preparations, have shown that supraphysiological doses of H_2S or H_2S donors evoked type I cell depolarisation (130) and $[Ca^{2+}]_i$ elevation (130, 131), and these effects appear to be quantitatively similar to those of hypoxia. Coupled to the type I cell excitation is the

capacity for exogenous H_2S to augment the chemoafferent discharge frequency, a response which is dependent on Ca^{2+} influx and neurosecretion of ATP and ACh (127, 132).

The precise mechanism of H_2S induced excitation is still to be clarified. H_2S attenuates type I cell TASK-like (130) and BK_{Ca} (127) currents in a manner that resembles the actions of hypoxia. In contrast to CO, the H_2S mediated depression of BK_{Ca} function appears not to involve specific interaction within the C-terminal domain (133), although an alternative binding site has not been identified.

Perhaps more importantly, is the suggestion that the exogenous concentrations of H_2S required to stimulate type I cells may be sufficient to impair mitochondrial function (130). H_2S is a recognised mitochondrial inhibitor characterised by its capacity to bind to and deactivate complex IV (134). It has been revealed that exogenous application of H_2S to dissociated type I cells, at concentrations equivalent to those that produce chemostimulation, significantly augmented NADH autofluorescence and increased Mg^{2+} fluorescence (130). These observations are consistent with a H_2S induced run-down in mitochondrial electron transport and a reduction in $[ATP]_i$. Therefore, a significant proportion of CB activation, caused by exogenous H_2S, probably occurs secondary to a reduction in mitochondrial ATP synthesis and cellular energy status.

A number of key investigations have examined whether endogenous generation of H_2S is necessary to activate the CB in hypoxia. The presence of CSE in mouse and rat type I cells has been confirmed by immunohistochemical techniques (127, 131, 132). Expression of CBS has also been reported in rat (133), mouse (127) and cat (135) type I

cells. In rats and mice it has been shown that CB H_2S production significantly increased in hypoxia (131, 132). Functionally, inhibitory targeting of CBS is reported to have abolished the hypoxic ventilatory response *in vivo* (127). On isolated CB tissue, hypoxia induced elevations in type I cell $[Ca^{2+}]_i$, dopamine secretion and chemoafferent discharge frequency, have all been shown to be diminished by pharmacological deactivation of CSE (131, 132).

More conclusive evidence has been presented in studies utilising transgenic KO models. Mice deficient in CSE ($CSE^{-/-}$) were observed to have an impaired ventilatory response to hypoxia (132). On *in vitro* CB preparations isolated from these mice, hypoxia evoked augmentations in $[Ca^{2+}]_i$, DA secretion and chemoafferent activity were all powerfully attenuated and this apparent down-regulation in hypoxic sensitivity was associated with a complete absence of hypoxia stimulated CB H_2S synthesis (131, 132).

Collectively, these data indicate that although the precise downstream target(s) of H_2S are unknown, endogenous H_2S generation in the type I cell is physiologically active and is necessary for evoking full CB stimulation in hypoxia.

1.5.3 Regulation of the carotid body response to hypoxia by nitric oxide

NO is considered to be an important chemical mediator in CB sensory chemotransduction. In contrast to CO and H_2S, the majority of evidence suggests that NO only has an inhibitory impact on CB activity.

In CB tissue, non-specific nitric oxide synthase (NOS) expression has been identified in both the efferent autonomic nerve bundles and in distal arterioles and capillaries (136-

138). Functionally, non-selective antagonism of NOS stimulates dose dependent increases in CSN discharge frequency, *in vitro*, indicative of a basal inhibitory action of NO (136). Similar agents also enhance the chemoafferent response to hypoxia, *in vitro* (138, 139). NO donors however, significantly attenuate chemoafferent activity and suppress ACh release in hypoxia (140-142).

Two isoforms of NOS have been proposed as contributing to NO generation within the CB; NOS-1 and NOS-3. Utilising transgenic mice, a number of studies have directly examined the functional significance of these NOS isoforms on regulating CB responses to hypoxia. NOS-1 homozygous KO mice were observed to have an exaggerated hypoxic ventilatory response compared with wild type littermates (143). In addition, exposure to 100% O_2 had a more pronounced inhibitory effect on ventilation and on phrenic nerve activity in the NOS-1 mutant mice, consistent with an up-regulation of basal chemoafferent discharge frequency. These observations suggest that NO derived selectively from NOS-1 inhibits chemoafferent activity in both normoxia and hypoxia.

In a similar study, mice deficient in NOS-3 appeared to have an impaired hypoxic ventilatory response and a diminished reflex increase in phrenic nerve activity upon exposure to cyanide (144). Delivery of 100% O_2 to these NOS-3 KO mice caused a smaller reduction in respiratory drive compared to wild type controls. These data perhaps support a role for NO derived from NOS-3 in excitatory regulation of CB activity. However, the authors proposed that chronic depletion of NOS-3 activity in the vascular endothelial cells would have evoked systemic vasoconstriction. It was hypothesised that vasoconstriction of the arterial supply to the CB would have subjected the organ to chronic hypoxia from birth. Chronic neonatal hypoxia is associated with an impairment of

the CB tissue to develop hypoxic chemosensitivity (45, 145). Using this explanation, the authors considered the attenuation of CB activity in these NOS-3 KO mice to be a consequence of chronic hypoxia rather than the loss of a direct stimulatory effect of NO derived from NOS-3. Although plausible, this conclusion might be questioned since quantitative analysis also revealed that CBs isolated from the NOS-3 mutant mice exhibited no significant increase in type I cell number compared with wild type controls. This suggests an absence of significant type I cell hyperplasia, a condition that is highly characteristic of chronic hypoxia (146, 147).

It is becoming more apparent that NO is capable of modifying type I cell function by acting on various targets in different cellular compartments. In dissociated type I cells it has been demonstrated that sodium nitroprusside (NO donor) depressed whole cell Ca^{2+} channel current and this action was attributed to a mechanism of NO mediated channel S-nitrosation (148). It has also been reported that type I cell BK_{Ca} current was enhanced by exogenous NO donors, but this effect was shown to be dependent on an augmentation in cGMP and PKG signalling (149, 150).

It has been ascertained that hypoxia leads to an elevation in the type I cell NO concentration (83). Since NOS-3 but not NOS-1 is localised in the type I cell (83), it seems logical to suggest that intracellular NO production is derived specifically from NOS-3 activity. Interestingly, by use of NO selective fluorescent dyes, Yamamoto et al. have been able to detect measurable amounts of NO in the mitochondrial membranes of type I cells in hypoxia. It is universally accepted that NO restricts mitochondrial energy metabolism through competitive binding with O_2 at the Cu_B/haem a_3 binuclear centre in complex IV (151). In the CB, this potential effect of NO on modifying the mitochondrial

function has not been investigated. However, in contrast to the vast majority of studies, it has been revealed that some NO donors may elevate the chemoafferent discharge frequency in normoxia (141). The reason for this contradictory finding is unclear but, as with CO, NO may have an additional and as yet uncharacterised excitatory impact on CB function and this may be mediated through the mitochondria.

Whether or not physiological concentrations of NO acutely or chronically alter type I cell mitochondrial energy metabolism remains an important area for consideration, especially given the close association between mitochondrial dysfunction and CB hypersensitivity in certain disease states (2).

1.5.4 Enzymatic nitric oxide generation from nitrite

Nitrite (NO_2^-) was originally thought to be an inert end product of NO metabolism that was of little/no functional significance in mammalian physiology. More recently, NO_2^- has been identified as an important mediator of systemic and pulmonary vasodilatation in rodents and in humans (152-155). Furthermore, pre-administration of NO_2^- has been shown to have cytoprotective effects during ischaemia-reperfusion injury in cardiac, renal, hepatic and brain tissue (156-158). As a consequence, NO_2^- biochemistry is currently receiving attention due to its potentially high level of biological and clinical significance.

The majority of the observed physiological actions of NO_2^- are thought to be a direct consequence of NO_2^- reductase activity and generation of NO. NO_2^- is now widely regarded as the largest biological store of NO in mammals, with tissue NO_2^- concentrations ranging from approximately 0.5-25 μM (159). Several enzymatic NO_2^-

reductases have been identified including deoxyhaemoglobin in blood, deoxymyoglobin, xanthine oxidoreductase, NOS-3, aldehyde dehydrogenase and carbonic anhydrase in the systemic and pulmonary vasculature, deoxymyoglobin in the heart and deoxyneuroglobin in brain and other neuronal tissues (155, 160-167). Of these the most widely characterised NO_2^- reductases by far is the family of haem-globins composed of haemoglobin, myoglobin and neuroglobin. Single electron reduction of NO_2^- in the presence of a proton and the deoxygenated ferrous haem-group leads to the formation of NO, as shown in the reaction below (in this case with myoglobin);

$$NO_2^- + \text{deoxyMb}(Fe^{2+}) + H^+ \rightarrow NO + \text{metMb}(Fe^{3+}) + OH^- \quad (168)$$

Since the reaction requires a proton and is favoured in the presence of the deoxygenated form of the ferrous haem group, the rate of NO generation is dependent on PO_2 and on pH. It has been reported that greater than 80% of NO_2^- reduction was inhibited in the presence of only 0.5% O_2 when measuring the rate of NO_2^- reduction in liver, red blood cell and heart tissue samples *in vitro* (169). In a different investigation, an acidic buffered (pH 6.6) solution increased the rate of NO_2^- reduction and NO production *in vitro* and this was coupled with a rise in the degree of aortic vessel relaxation (170).

1.5.5 Mitochondrial nitrite reduction as a novel mechanism for NO generation

In addition to NO_2^- reduction by cytosolic enzymes it has been proposed that NO_2^- reductase activity within the mitochondria may contribute to local mitochondrial NO production and in turn allow for direct modulation of mitochondrial oxidative phosphorylation and O_2 consumption (168). The earliest study investigating this novel

function of the mitochondria was performed using isolated hepatic mitochondria and demonstrated that under anoxic conditions addition of exogenous NO_2^- led to the subsequent generation of NO (171). Complex III (171), complex IV (172, 173) and cytochrome c (174) have all since been proposed as potential mitochondrial NO_2^- reductases and the overall mitochondrial NO generation from NO_2^- may have varying contributions from each source.

In the case of NO generation from NO_2^- by cytochrome c oxidase, a mechanism of single electron reduction at the site of the reduced haem (Fe^{2+}) group has been advocated, similar to that observed in the presence of the cytosolic haem-globin proteins (see above reaction) (172). As in the cytosol, mitochondrial reduction of NO_2^- is considered to be exquisitively sensitive to O_2 and pH and is favoured in hypoxia and acid (171, 172, 174). In contrast to cytosolic enzymatic NO_2^- reduction, that can occur at physiological (μM) concentrations of NO_2^-, mitochondrial NO_2^- reduction seems only to occur at supraphysiological (mM) NO_2^- concentrations (172-174). This may in part be due to the limited mitochondrial uptake of exogenous NO_2^-. Castello and colleagues found that only 10% of exogenous NO_2^- added to the superfusate was subsequently internalised into the mitochondria (172). It has been speculated that very high local concentrations of NO_2^- may exist within the mitochondria, but as yet there is no evidence to substantiate this claim (168). Although intriguing, a definitive physiological role for NO formed from endogenous NO_2^- at the mitochondria remains to be verified.

1.5.6 Modification of mitochondrial oxidative phosphorylation by nitrite

Cellular NO_2^- reductase activity leading to the local generation of NO has the potential to evoke post-translational changes in mitochondrial proteins through nitrosation of amino

acid thiol (R-S-H) and amine (R-N-H_2) groups (159). Rises in NO produced from NO_2^- in hypoxia may also promote the formation of haem-NO complexes (175, 176). Inhibitory regulation of mitochondrial oxidative phosphorylation through binding of NO to the Cu_B/haem a_3 binuclear centre within complex IV is well characterised (177, 178). The impairment of complex IV activity by NO is considered to be O_2 dependent and rapidly reversible with a K_i of approximately 60-150 nM NO at O_2 concentration of 5–10 μM (179).

NO derived specifically from NO_2^- has now been demonstrated to significantly diminish mitochondrial respiration via inhibitory targeting of complex IV (180). The degree of NO-haem complex formation and mitochondrial inhibition with NO_2^- appears to be highly dependent on PO_2 and only occurs in hypoxia (176, 180). In the absence of cytosolic NO_2^- reductases, mitochondrial activity is only depressed by supraphysiological NO_2^- concentrations, consistent with those shown to generate NO directly from the mitochondria (180).

Attenuation of mitochondrial function by NO_2^- may also be dependent on post-translational modification of mitochondrial proteins. Treatment of isolated cardiac mitochondria in anoxic conditions with NO_2^- has been shown to produce dose dependent reductions in the respiration rate through complex I and this was coupled to a decrease in complex I ROS generation upon reoxygenation (181). In the same investigation, R-SNO levels, measured by chemiluminescence, were augmented in the mitochondrial fraction containing complex I following NO_2^- treatment, leading authors to conclude that a dampening of total mitochondrial electron flux was a direct consequence of complex I

protein S-nitrosation. A summary of the mitochondria being both metabolisers and targets of NO_2^- is presented in Figure 1.3.

NO_2^- is recognised as a novel and rapidly reversible inhibitor of mitochondrial electron transport in various tissues and acts primarily through generation of NO. NO_2^- reductase activity has never been detected within the CB. In contrast to other NO donors used previously in the CB, NO_2^- may be able to form NO exclusively in the type I cell mitochondria. In this way, NO_2^- may be a useful agent to examine whether NO can adjust the CB hypoxic sensitivity by selective modification of type I cell mitochondrial function.

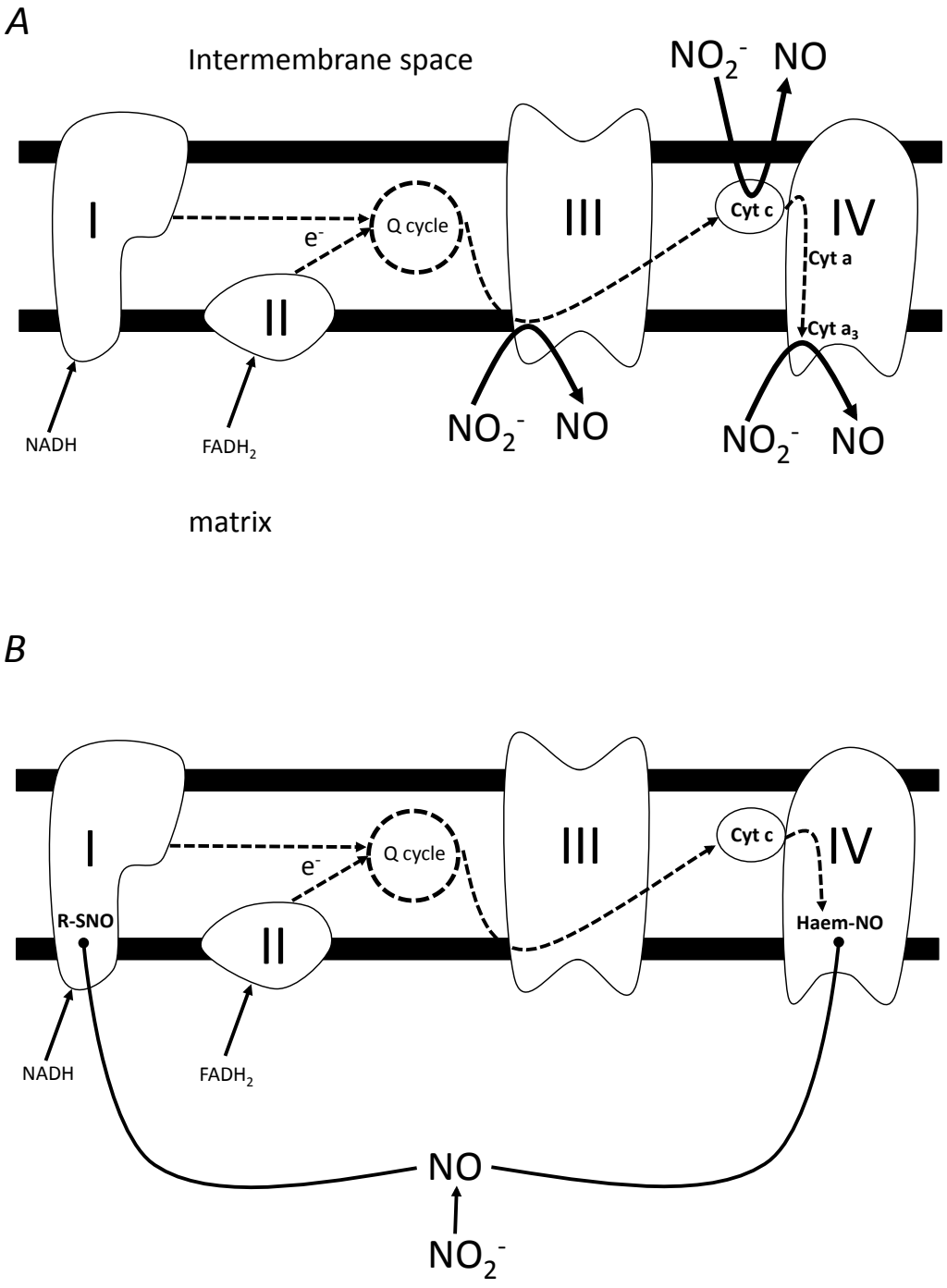


Figure 1.3 The mitochondria as metabolisers and targets of nitrite.
A) Supraphysiological (mM) concentrations of nitrite (NO_2^-) can be reduced at three sites within the mitochondria to generate NO. These sites are; complex III, complex IV and cytochrome c. NO_2^- reduction is favoured in acidic and hypoxic conditions and requires continuous mitochondrial substrate supply ($NADH/FADH_2$) and electron flux (indicated by the dotted line). B) The NO generated from NO_2^- reduction has the potential to impair mitochondrial electron transport by nitrosation of amino acid thiol groups in complex I (generating R-SNO groups) or by forming haem-NO complexes in complex IV. Adapted from Shiva, 2010 (168).

1.6 Mechanisms of hypercapnic chemotransduction in the carotid body

1.6.1 Established hypercapnic chemotransduction processes in the carotid body

Approximately 30-50% of the reflex ventilatory response to arterial hypercapnia is mediated through direct stimulation of the CB chemoreceptors (182, 183), with the remaining contribution arising from chemoreceptors located in the CNS. Complete silencing of the CSN chemoafferent output, using a local hyperoxic and hypocapnic solution, has been shown to severely depress the reflex ventilatory response to systemic hypercapnia to only 20% of control (184). Therefore, in addition to the reflexes arising directly from hypercapnic CB excitation, maintenance of a definitive CB chemoafferent input into the CNS seems to have a role in establishing the acute hypercapnic sensitivity of the central chemoreceptors.

The CB chemoafferent response curve to increasing P_aCO_2 (at constant P_aO_2) has a sigmoid shape with a linear increase in discharge frequency identified over a range of approximately 25-80 mmHg P_aCO_2 (40, 41, 185). At a higher or lower P_aCO_2 the discharge frequency tends to plateau (40, 186). A similar correlation between superfusate PCO_2 and chemoafferent discharge has been detected in the intact CB preparation *in vitro* (46). The chemoafferent response to a single level of hypercapnia is rapid, peaking within 2-4 s, and then it adapts quickly, within 10 s, to a slightly lower steady state frequency (187) that is maintained for up to 240 minutes, with very little further depression (186).

Transduction of the hypercapnic stimulus into a functional chemoafferent neural signal involves many of the same processes associated with hypoxic sensing. These include, type I cell depolarisation, Ca^{2+} influx and neurosecretion (18). As with hypoxia, the vast majority of the $[Ca^{2+}]_i$ rise in response to hypercapnia is dependent on type I cell depolarisation and Ca^{2+} influx through L-type Ca^{2+} channels (188). The mechanism behind the small residual rise in Ca^{2+} independent of L-type Ca^{2+} channel activity has not been clearly defined, but evidence implies that it may involve Ca^{2+} entry through the P/Q type Ca^{2+} channels (189) or acid induced reverse activation of the Na^+/Ca^{2+} exchanger (53).

The hypercapnic induced elevation in $[Ca^{2+}]_i$ is essential for the secretion of stored neurotransmitters that stimulate post-synaptic action potential generation in the adjacent chemoafferent fibres. As in hypoxia, the two critical excitatory neurotransmitters are considered to be ACh and ATP, as evidenced by the concurrent block of both nicotinic ACh receptors (nAChR) and purinergic P2 receptors causing a complete obliteration of the chemoafferent response to hypercapnia (190). The release of DA has also been reported under hypercapnic conditions, although this most likely has an autoinhibitory, rather than excitatory function (191).

1.6.2 Linking hypercapnia with type I cell depolarisation; a role for carbonic anhydrase and H^+

The precise signalling mechanism that couples high extracellular PCO_2 with type I cell depolarisation remains unresolved. A number of studies have proposed that the crucial step in the transduction process is the intracellular hydration of CO_2 to form H_2CO_3 followed by dissociation into HCO_3^- and H^+ (190, 192, 193). The rate of CO_2 hydration is

rapidly increased in the presence of carbonic anhydrases (CAHs) (194) and the function of these enzymes within the type I cell may be functionally relevant in mediating the response to hypercapnia. Expression of CAHs in the CB has been widely reported (195) (196) and the most detailed analysis has revealed the presence of CAHI, CAHII and CAHIII isoforms within the type I cell (197).

On a functional level, inhibition of CAH using acetazolamide has been associated with a delayed and a depressed CB chemoafferent response to hypercapnia *in vitro* (192), consistent with a reduction in the rate of intracellular CO_2 hydration. The absolute level of steady state suppression seems to be dependent on the CB preparation used; CAH inhibition almost completely ablated the frequency response to hypercapnia in petrosal neurones co-cultured with type I cell clusters (190), but evoked a much less severe reduction in chemoafferent frequency in the intact perfused CB preparation (198). *In vivo*, it has been observed that chemoafferent elevations in hypercapnia were significantly attenuated by acetazolamide, a membrane permeable CAH inhibitor, but not by benzolamide, a membrane impermeable CAH inhibitor (199).

Perhaps the most obvious explanation for the impact of CAH activity on type I cell excitability would be the increase in $[H^+]_i$ leading to a fall in intracellular pH (pH_i). A rise in extracellular PCO_2 in isohydric conditions (achieved by concurrently raising the superfusate HCO_3^-) causes only a small transient intracellular acidosis that gradually recovers over 4-8 minutes (200). This restoration of pH_i is probably a consequence of an increased rate of H^+ extrusion. The maintenance of a steady state acidic pH_i in hypercapnia has therefore been proposed as being dependent not only on the intracellular hydration of CO_2 by CAH and generation of H^+, but also on a concurrent

extracellular acidosis and a depression of type I cell H^+ extrusion (200). In hypercapnia, a clear linear correlation between extracellular pH (pH_o) and type I cell pH_i has been described over the physiological range (200). Impairment of H^+ extrusion following a decrease in pH_o may be due to the H^+ induced inhibition of the H^+/Na^+ exchanger and up-regulation of the HCO_3^-/Cl^- exchanger and the activity of the non-specific (HCO_3^- conducting) anion channel (200, 201).

If secondary acidosis is central to the process of CB CO_2 sensing then there must exist a mechanism of acid induced cellular excitation. A number of channels expressed in the type I cell are recognised as being sensitive to alterations in pH and modification of their activity may directly lead to type I cell depolarisation. Severe acidosis (pH 6.4) directly attenuates the background TASK-like current and the same stimulus can evoke type I cell depolarisation (27). A similar reduction of the background K^+ leak current during hypercapnic stimulation has been demonstrated (188). Accordingly, TASK-1 KO mice were reported to have a blunted ventilatory response to hypercapnia (202). Subsequent *in vitro* analysis identified an impaired augmentation of chemoafferent activity in response to hypercapnia in CBs isolated from TASK-1 KO mice, suggestive of a diminished CO_2 sensitivity. However, these findings were in contrast to an earlier investigation that described a maintained DA secretory response to hypercapnia in CB slices harvested from TASK-1/3 deficient mice (203). Thus, a role for TASK channels in CB PCO_2/H^+ remains to be verified.

In addition to TASK, it has been established that the outward K^+ current conducted through the BK_{Ca} channel is suppressed by acidosis (25, 201). Emerging evidence also indicates that inward Na^+ (204) and Cl^- (205) currents are activated in type I cells in

response to a fall in extracellular pH. The relative contribution of all of the currents described in mediating type I cell depolarisation in hypercapnia is still to be fully characterised.

1.6.3 Effects of CO_2 on carotid body function that are independent of pH changes

If CB activation in hypercapnia is purely a consequence of intracellular acidosis, (which in itself is dependent on simultaneous extracellular acidosis), then it might be expected that responses to hypercapnia when the extracellular pH is maintained at 7.4 are either severely diminished or absent. However, it has been reported that isohydric hypercapnia can evoke increases in type I cell Ca^{2+} current (206), neurotransmitter release (191) and activation of the CSN afferents (190). In these latter two studies the magnitude of the response in isohydric hypercapnia was not as great as that observed in acidic hypercapnia but a large residual response was still preserved. Isohydric hypercapnia does generate a small transient intracellular acidosis in type I cells (200), but the size of these responses is still conceivably greater than would be expected if hypercapnic chemotransduction was completely dependent on intracellular acidosis. This indicates that there may be excitatory mechanisms induced by hypercapnia that are independent of concurrent acidosis.

The elevation in type I cell Ca^{2+} current identified in isohydric hypercapnia (described above) was shown to be mimicked by cAMP analogues and abolished by inhibition of PKA (206). This suggests that cAMP production and subsequent PKA activation is functionally relevant in CB hypercapnia stimulus response coupling. The authors hypothesised that cAMP generation may be a consequence of the increase in HCO_3^- production from CAH, leading to the direct stimulation of the HCO_3^- sensitive soluble

adenylate cyclase (sAC). The expression of sAC mRNA in type I cells has recently been confirmed (207) and CB cAMP levels do increase in hypercapnia (208). However, it remains to be seen whether sAC is functionally active in the CB and has a specific role in the chemotransduction process of hypercapnia.

1.6.4 CO_2-O_2 interactions in the carotid body

A considerable quantity of evidence suggests that hypoxic and hypercapnic stimuli interact at the level of the CB. The *in vivo* chemoafferent response to hypercapnia is highly dependent on the P_aO_2 (185, 209). Although a significant elevation in frequency upon hypercapnic stimulation occurs at any PO_2, the absolute rise in frequency per mmHg increase in PCO_2 is strongly diminished by arterial hyperoxia (185, 209).

It has also been established that the CB response to hypoxia is sensitive to variations in CO_2. Detailed analysis of CB chemoafferent activity *in vitro* revealed that hypercapnia caused a significant 'right shift' of the CB hypoxic response curve (46). This implies that hypercapnia reduces the hypoxic threshold required for response initiation. In the same investigation, the absolute impact of hypercapnia on discharge frequency was augmented in hypoxia, again emphasising the synergy between these two stimuli (46). The precise site of O_2-CO_2 interaction remains elusive but the transduction pathways involved in hypoxia and hypercapnia signalling have much in common (18). Since the $[Ca^{2+}]_i$ elevation in response to hypercapnia is enhanced in hypoxia (210), the convergence of these two signalling cascades is most likely to occur within the type I cell, at or before the generation of $[Ca^{2+}]_i$. Further evaluation of the interaction between these two stimuli and the magnification of CB output on the control of respiratory and

cardiovascular reflexes could be of importance given the close association between CB hyperexcitability and disease (2).

1.7 The carotid body in glucose sensing

1.7.1 General overview of the properties and mechanisms of some glucose sensitive tissues

Glucose homeostasis in mammals is an essential process that allows for continuous maintenance of glycolysis. Counter regulatory responses to hyper or hypoglycaemia are dependent on a number of specialised glucose sensitive cells that respond to glucose concentrations over a range that fails to evoke stimulation in all other cell types. Whether the CB type I cell is one of these physiological glucose sensors, currently, appears equivocal. Experiments performed in this thesis aim to examine the direct sensitivity of the CB to low glucose and to unify the apparent contradictory observations that have been previously reported in the literature.

Pancreatic β-cell

The most commonly studied mammalian glucose sensor is the pancreatic β-cell. Insulin secretion induced by hyperglycaemia is coupled tightly with changes in the rate of glucose metabolism and ATP generation (reviewed in (211)). An increase in $[ATP]_i$ through glycolysis and oxidative phosphorylation reduces K_{ATP} channel activity. This promotes cellular depolarisation (212, 213), an increase in voltage dependent Ca^{2+} influx (214) and insulin secretion.

The primary regulator of glycolysis in the β-cell is glucokinase (hexokinase IV). Glucokinase has an exceptionally low affinity for glucose; it has a K_m of approximately 8 mM glucose, which is far higher than other hexokinases that are in the μM range (215, 216). This makes it exquisitively sensitive to fluctuations in glucose concentrations across the entire physiological range. In isolated β-cells it has been demonstrated that elevations in glucose metabolism and insulin secretion in response to hyperglycaemia were associated with an increase in glucokinase expression and activity (217). A later report showed that a 70% reduction in β-cell glucokinase expression was associated with a diminished the rate of pancreatic insulin secretion in response to hyperglycaemia (218).

The glucose transporter GLUT-2 may also control glucose utilisation in the β-cell by modifying the rate of glucose uptake. GLUT-2 is highly expressed in the pancreatic β-cell and its K_m for glucose (approximately 17 mM) is far higher than that reported for GLUT-1, GLUT-3 and GLUT-4 (219). Observations made by Johnson et al. indicate that extracellular glucose uptake into the β-cell is directly dependent on the level of GLUT-2 activity (220). Complete knockdown of *glut2* gene expression in mice has been shown to dramatically reduce the degree of the glucose induced insulin secretion (221). Thus, the physiological acute glucose sensitivity of the β-cell appears to be critically dependent on the expression and activity of both GLUT-2 and glucokinase.

Glucose sensitive neurones

Glucose sensitive neurones in the mammalian brain are found predominantly in the arcuate nucleus (ARC) and the lateral hypothalamic region (LH) (222-224). These neurones are either acutely stimulated (GE) or inhibited (GI) by rises in glucose

concentrations (reviewed in (225)). Despite the lack of specific cell markers there is some degree of association between both GE and GI neurones and the presence of glucokinase, with 64% of GE neurones and 43% of GI neurones expressing glucokinase mRNA (226-228).

As in the pancreatic β-cell, the majority of GE neurones are activated subsequent to the inhibition of K_{ATP} channels (229, 230) following a glucokinase mediated up-regulation in glycolysis and ATP synthesis (227). Some GE neurones seem to be stimulated via glucose mediated activation of the glucose, Na^+ co-transporter and this mechanism is independent of an elevation in glycolysis (231).

The means by which GI neurones are stimulated in response to low glucose is less well defined. A reduction in glucose metabolism appears to be significant in the coupling process in some GI neurones. In these neurones glucokinase is central to the regulation of glycolysis and ATP synthesis (reviewed in (232)). It has been reported that acute activation of glucokinase by Compound A significantly reduced glucagon secretion in response to insulin induced hypoglycaemia (233). In the same study, chronic knock down of glucokinase mRNA increased the level of adrenaline release during hypoglycaemia. An earlier study showed that pharmacological inhibition of glucokinase rapidly increased $[Ca^{2+}]_i$ in isolated GI neurones (229), indicative of a reduction in glucokinase activity coupling directly to GI neurone excitation.

Activation of some GI neurones is considered to be conferred through an increase in the AMP:ATP ratio and activation of AMPK subsequent to a fall in glycolysis and ATP synthesis. Intracerebral venous administration of 2-deoxyglucose (glycolysis inhibitor)

has been shown to increase AMPK activity in hypothalamic neurones, and this was associated with an elevation in food intake (234). In a later study, microinjection of the AMPK inhibitor Compound C into the ARC, attenuated the level of adrenaline and glucagon secretion in response to hypoglycaemia (235). Observations made on isolated brain slices have also revealed that exogenous application of AICAR stimulated GI neurones in a manner that was comparable to low glucose (236). Alternatively, it has been proposed that other GI neurones may respond to low glucose following a run-down in the Na^+/K^+ ATPase (237). As with AMPK activation, this is probably due to a low glucose mediated reduction in glycolysis and ATP synthesis.

1.7.2 A role for the carotid body in systemic glucose homeostasis

The emerging consensus is that the CB is involved in glucose homeostasis. Direct pharmacological stimulation of the CB *in vivo* evokes a reflex elevation in arterial glucose concentration (238). This increase is reliant on a heightened chemoafferent input into the NTS that in turn leads to the augmentation of adrenaline secretion from the adrenal medulla and an increase in hepatic glucose release into the systemic circulation (238, 239).

Koyama and colleagues have reported that during insulin induced hypoglycaemic clamp, the rate of glucose infusion required to maintain the hypoglycaemic level was elevated in dogs following CB resection (240). These dogs also exhibited a reduced level of endogenous hepatic glucose production. In humans, throughout a similar insulin induced hypoglycaemic clamp, the rate of glucose infusion necessary to maintain the serum glucose at 3.3 mM was increased following proposed silencing of chemoafferent activity by hyperoxia (241). Taken together, these data indicate that the CB is stimulated in

hypoglycaemia and contributes to the counter regulation required to restore a normal plasma glucose concentration.

1.7.3 Altered chemosensitivity and ventilatory responses in hypoglycaemia

A number of investigations have evaluated potential changes in peripheral chemoreceptor sensitivity and ventilation during hypoglycaemia. Bin-Jaliah et al. demonstrated that insulin induced hypoglycaemia augmented minute ventilation (V_E) and the rate of O_2 consumption (VO_2), so that the V_E:VO_2 ratio remained constant (242). The precise matching of ventilation with the elevation in metabolism during hypoglycaemia was shown to be critically dependent on the preservation of CB chemoafferent activity. In a follow up article, a similar insulin induced hypoglycaemic clamp significantly augmented the CB sensitivity to hypercapnia (243). Since exposure of the intact *in vitro* CB to 2 mM glucose did not directly potentiate chemoafferent responses to hypercapnia, the authors proposed that the elevation of CB CO_2 chemosensitivity observed *in vivo* was an indirect consequence of systemic hypoglycaemia, most likely related to a simultaneous increase in metabolic rate.

In a less severe model of hypoglycaemia, induced by fasting the animals for 12 hours, it has been observed that a 25% reduction in basal serum glucose had no effect on normoxic ventilation, O_2 consumption or CO_2 production (244). During acute hypoxia there was no difference in the increase in minute ventilation between fed and fasted animals. This suggests that mild hypoglycaemia, at a level that does not alter whole body metabolism, does not augment CB chemosensitivity.

In humans, it has been reported that severe hypoglycaemia (2.8 mM) increased basal ventilation and augmented ventilatory responses to hypoxia (245). These effects were coupled with rises in serum glucagon, adrenaline, noradrenaline and cortisol concentrations. Interestingly, immediately following the restoration of a normal plasma glucose concentration, the ventilatory response to hypoxia remained elevated. Analysis of serum hormones revealed that noradrenaline and cortisol had not returned to baseline. In view of these findings, it is possible that the CB activation in hypoglycaemia is mediated indirectly through the release of counter regulatory endocrine or neuroendocrine factors that may also be partially responsible for elevating whole body metabolism.

1.7.4 Acute sensitivity of the carotid body to low glucose *in vitro*

A number of studies have assessed the direct low glucose sensitivity of *in vitro* CB tissue in order to determine whether it can act as a physiological glucose sensor in the whole animal (242, 246-249). So far, the findings from these investigations have been unable to establish a clear relationship between low glucose and CB activation. Several clear discrepancies have been reported and at present the direct low glucose sensitivity of the CB is ambiguous. It has been suggested that the reason for these disparities may be due to dissimilar isolation procedures or tissue incubation conditions utilised throughout the different investigations (250). In addition, Zhang et al. have proposed that the explanation for the absence of low glucose sensitivity reported in certain CB preparations was purely a consequence of the high PO_2 levels used (248). A summary of the current evidence regarding the direct low glucose sensitivity of the CB is described in this section.

The freshly isolated intact CB appears to be acutely unresponsive to fluctuations in the superfusate glucose concentration. Bin-Jaliah and colleagues showed that exposure of the CB to 2 mM glucose failed to acutely enhance the CSN chemoafferent activity (242). Consistent with these findings, Conde et al. reported that ^{3}H-CA neurotransmitter release was not acutely augmented even by 1 mM glucose in normoxia or moderate hypoxia (247). In addition, complete glucose deprivation does not acutely alter the CB secretion of the two primary excitatory neurotransmitters; ATP (247) and ACh (251). An increase in ^{3}H-CA has been observed but only after the intact CB has been exposed to glucose deprivation for at least 40 minutes (247).

A similar lack of intrinsic low glucose sensitivity has been identified in freshly dissociated type I cells. The background TASK-like channel current was shown to be completely unaffected by glucose deprivation (252). The recently characterised ATP sensitive K^{+} (K_{ATP}-like) current (distinguishable from TASK) is also reported to be completely insensitive to the removal of superfusate glucose (252). Finally, dissociated type I cells, experimented on within hours of CB isolation, exhibit no degree of $[Ca^{2+}]_{i}$ elevation in response to removal of glucose from the superfusate (249). This is consistent with the absence of low glucose induced cellular activation.

In contrast with the studies described above, it is also accepted that several long term culture CB preparations are acutely stimulated by low glucose. In the CB slice (incubated for 24-48 hours prior to experimentation), low glucose activates the type I cell leading to a rapid increase in the rate of DA release (246, 253). Low glucose also enhances the CB slice secretory response to hypoxia (246). In common with hypoxia, DA secretion in response to glucose deprivation is dependent on Ca^{2+} influx through

voltage gated Ca^{2+} channels (253). In the 6-10 day old CB co-culture preparation (type I cell clusters co-cultured with chemoafferent petrosal neurones) low glucose evoked an increase in afferent fibre spike frequency that was equivalent in amplitude to that induced by hypoxia (248). The same concentration of glucose significantly potentiated the response to hypercapnia, indicative of a significant degree of stimulus interaction.

In both the CB slice and co-culture preparations, activation in response to physiological levels of low glucose was induced by reducing the superfusate PO_2 to approximately 90 mmHg (246, 248). This raises the idea that the CB sensitivity to low glucose may be dependent on the background level of O_2 tension. Experiments by Bin-Jaliah et al. (242, 243) utilising the intact preparation were performed in hyperoxia (superfusate PO_2 of approximately 400 mmHg), and it has been suggested that this high O_2 tension may have acted to conceal the low glucose sensitivity of the tissue (248).

Alternatively, it has been speculated that the intrinsic low glucose sensitivity of the CB slice and co-culture preparations is a consequence of an alteration in metabolic status following prolonged tissue incubation (250). Some support for this idea has come from a recent article demonstrating the induction of small $[Ca^{2+}]_i$ responses to glucose deprivation following 24 hours of culture (249). However, in this same study, the rate of cellular DA secretion was still unaffected by glucose deprivation after the equivalent time of incubation, implying that the rise in $[Ca^{2+}]_i$ was not sufficient to elicit neurotransmitter release. Further evaluation of the CB response to low glucose is therefore required to fully unite the apparent contradictory findings that have been described in different CB preparations. Experiments performed in this thesis aimed to clarify whether the low

glucose sensitivity of the CB is dependent on an interaction with O_2 and/or is induced following long term tissue incubation *in vitro*.

1.8 Neurotransmission and neuromodulation in the carotid body

1.8.1 Acetylcholine and ATP

It is thought that the hypoxia induced release of excitatory neurotransmitters from the type I cell is crucial for the elevation in action potential generation in the chemoafferent petrosal neurones. This is primarily based on evidence that identified a lack of intrinsic O_2 sensitivity in petrosal neurones that were cultured in the absence of co-localised type I cells (254). In Ca^{2+} free media, rises in type I cell $[Ca^{2+}]_i$ and chemoafferent excitation in hypoxia are almost completely abolished (31). This indicates that Ca^{2+} evoked neurosecretion is necessary for post-synaptic action potential generation.

The majority of evidence suggests that the two main excitatory neurotransmitters synthesised and released from type I cells are ACh and ATP. Hypoxia stimulated secretion of ACh has been detected both *in vivo* (35) and *in vitro* (36). The type I cell exhibits positive immunoreactivity for a number of cholinergic markers including choline acetyl transferase (ChAT; ACh synthesising enzyme) (255), the vesicular ACh transporter (VChaT) (256) and acetylcholine esterase (AChE) (257). These findings imply that the type I cell is a primary source of hypoxia induced synaptic ACh generation.

Direct application of ACh to isolated chemoafferent petrosal neurones evokes a depolarisation that is coupled to the generation of a fast and rapidly desensitising inward

current (258). This current is mimicked by nicotine and abolished by hexamethonium (HEX; nAChR antagonist); thus strongly indicative of the presence of functional nicotinic ACh receptors (nAChR) on these petrosal neurones. Selective inhibitory targeting of nAChR *in vivo* using mecamylamine has been observed to attenuate the increase in chemoafferent firing frequency during hypoxia (259). In petrosal neurones co-cultured with type I cell clusters, *in vitro*, the rise in action potential frequency evoked by hypoxia is partially impaired by a number of different nAChR antagonists (72, 254). In contrast, a recent study has reported that in the intact CB preparation, a very high dose of mecamylamine (nAChR antagonist) completely ablated the chemoafferent response to ACh, but had little or no effect on the elevation induced by hypoxia (260). Although ACh is widely regarded as an important and functional excitatory neurotransmitter in the CB, its precise physiological role in post-synaptic action potential generation in hypoxia is perhaps not as definitive as originally thought.

Findings from the above investigations indicate that another excitatory neurotransmitter in addition to ACh must be secreted in order to attain peak chemoafferent frequencies upon CB stimulation. ATP is now widely acknowledged as this neurotransmitter. ATP is stored in secretory granules within the type I cell (71) and is released upon hypoxic stimulation secondary to Ca^{2+} influx through L-type Ca^{2+} channels (34). Immunohistochemical analysis has revealed the presence of $P2X_2$ and $P2X_3$ purinergic receptors in the chemoafferent nerve terminals adjacent to type I cell clusters (261). These receptors were co-localised, which is indicative of the formation of $P2X_2$-$P2X_3$ heteromultimers, similar to those detected in sensory neurones in the dorsal root ganglion (262). It has been demonstrated that exogenous application of ATP to isolated

chemoafferent petrosal neurones elicits a large inward current that slowly desensitises, consistent with the currents carried through $P2X_2$-$P2X_3$ heteromultimers (72, 262).

Intravenous infusion of the ATP analogue (α,β-methylene ATP) has been shown to induce significant hyperventilation in the anaesthetised rat and this was coupled with a marked escalation in CB chemoafferent activity (263). In unanaesthetised rats, suramin infusion (a non-specific P2 receptor antagonist) decreased the ventilatory response to hypoxia, primarily by impairing the reflex increase in respiratory frequency (264). In the CB co-culture preparation, *in vitro*, suramin application strikingly attenuated the hypoxia evoked rise in petrosal action potential frequency (72). Suramin also markedly reduces the CB sensitivity to hypoxia in the intact CB preparation (72, 260).

More conclusive evidence in support of the idea that ATP is an important excitatory neurotransmitter has been provided from studies utilising genetically modified mice. $P2X_2^{-/-}$ and $P2X_2$-$P2X_3^{dbl\ -/-}$ mice exhibit a suppressed hypoxic ventilatory response (265), and in intact CBs isolated from these mice, the peak chemoafferent response to hypoxia and the hypoxic sensitivity are both markedly diminished.

1.8.2 Dopamine and serotonin

The CB contains a large amount of stored catecholamines (CAs) in dense core vesicles, and for its mass the overall CA content is equivalent only to that seen in adrenal medullary tissue (266). The enzyme tyrosine hydroxylase (TH), that initiates the synthesis of CAs from tyrosine, has now emerged as a well established type I cell marker (267). Comparatively, dopamine (DA) is the most abundant CA in type I cells, forming more than 50% of the overall CA content (266, 268). Noradrenaline and

adrenaline account for the rest of the CB CA content and are produced at much lower levels (266, 268). Although it is known that DA is secreted from type I cells in abundance in hypoxia (33, 37), its action seems to be autoinhibitory rather than excitatory, providing the CB with a degree of inhibitory feedback control (269-271).

The inhibitory effects of DA appear to be mediated through activation of the D_2 receptor. Radio-ligand binding was implemented to originally identify the presence of numerous D_2 receptors throughout the whole CB tissue (272). More recently, D_2 receptor mRNA was detected in the type I cell (273). Direct application of DA to dissociated type I cells has been shown to attenuate the voltage dependent inward Ca^{2+} current (39). Hypoxia induced rises in Ca^{2+} are recognised as being depressed in the presence of specific D_2 receptor agonists (274). Similar D_2 receptor agonists were also reported to decrease ^{3}H-DA neurosecretion both in normoxia and in hypoxia (275). Importantly, this action was associated with a decrease in CB cAMP content under both conditions. Therefore, the inhibitory effects on DA are considered to be dependent on a D_2 receptor mediated reduction in the type I cell $[cAMP]_i$.

Serotonin (5-HT) is another monoamine that is stored in the type I cell (276). Unlike DA, 5-HT appears to be an excitatory neuromodulator of type I cell activity. It has been observed that the spontaneous depolarisations and action potentials generated in large clusters of type I cells (> 30 cells) were almost completely ablated by the 5-HT_2 receptor antagonist ketanserin (277). This led authors to suggest that endogenous release of 5-HT in normoxia established the basal type I cell electrical rhythm. The same group showed that the spontaneous action potential generation in type I cells in normoxia was mimicked by application of a DAG analogue (OAG), was attenuated by PKC inhibition

and was dependent on a reduction in background K^+ channel current (278). It has been reported that ketanserin does not reduce the CB chemoafferent response to hypoxia, suggesting that the excitatory actions of 5-HT are limited to normoxia and that 5-HT is not an essential neurotransmitter for CB activation by hypoxia (279).

1.8.3 Adenosine signalling in normoxic/hyperoxic conditions

Chapter 6 focuses on investigating whether adenosine has an important neuromodulatory function in mediating CB responses to hypoxia, mitochondrial inhibition and hypercapnia. Endogenously produced adenosine has often been regarded as an overlooked signalling molecule in the CB (9). A comprehensive understanding of the physiological role of adenosinergic signalling in establishing the overall sensitivity of the CB to physiological or pathological stimuli may allow for the development of additional therapeutic agents to modify CB excitability in certain pathologies. The following sections describe what is currently known about the actions of adenosine in the CB.

Exogenous adenosine administration has been shown to increase the chemoafferent discharge frequency in a dose dependent manner *in vivo* (280, 281) and *in vitro* (282, 283). CB activation by adenosine *in vivo* evokes an acute increase in respiratory frequency, tidal volume and minute ventilation that are dependent on A_2 receptor stimulation (284). In arterial normoxia, suppression of adenosine metabolism or uptake mimics the excitatory action of exogenous adenosine and causes acute respiratory stimulation. Since these ventilatory effects are abolished by CSN section, it has been proposed that adenosine is generated endogenously in the CB and mediates the basal chemoafferent activity and the peripheral component of respiratory drive in arterial normoxia (285).

The peripheral chemoexcitatory actions of adenosine on the CB function have also been detected in humans. Intra-aortic adenosine infusion was shown to increase minute ventilation in patients undergoing cardiac catheterization (286). In addition, exogenous adenosine application has been linked to an increase in muscle sympathetic neuronal firing frequency (287). A later study demonstrated that augmentation of endogenous adenosine signalling by dipyridamole (a re-uptake inhibitor), during room air breathing, augmented sympathetic activity and increased ventilation in all subjects (288). Since these potentiations were blunted in hyperoxia it was suggested that the adenosine mediated increase in sympathetic firing frequency was attributable to a concurrent elevation in CB chemoafferent activity.

Generation of adenosine in the synapse between the CB type I cell and the adjacent petrosal afferent neurone may be the result of extracellular ATP catabolism. ATP is stored in vesicles in type I cells (71) and released in normoxia/hyperoxia (34). Extracellular ATP and ADP can be converted to AMP in the presence of ectonucleoside triphosphate diphosphohydrolyase 1 (CD39), and then into adenosine by the membrane bound ecto-5'-nucleotidase (CD73) (289). Alternatively, adenosine can be produced from intracellular ATP catabolism and released into the synapse through the bidirectional equilibrative adenosine transporter (ENT) (290). Adenosine derived from the whole CB has been detected under normoxic conditions *in vitro* (11). The level of adenosine recovered in normoxia was depressed in the presence of the CD73 inhibitor α,β-methylene ADP (AOPCP) but not by the ENT blocker S-(4-nitrobenzyl)-6-thioinosine (NBTI), indicating that adenosine is predominately produced in normoxia through extracellular ATP catabolism mediated by CD39 and CD73 activity.

1.8.4 Adenosine generation and signalling in hypoxia

Adenosine has been advocated as an important substance in neuromodulation of the peripheral chemoreceptor response to hypoxia. This was first evidenced by observations *in vivo* in the cat demonstrating that the characteristic increase in CSN discharge frequency in hypoxia was attenuated by non-selective adenosine receptor blockade (291). Inhibition of adenosine receptors with 8-(p-sulfophenyl) theophylline (8-SPT) in rats also decreases, but does not abolish, the acute phase of the hypoxic ventilatory response (292). In humans, adenosine infusion at a level that does not alter baseline MABP increases basal minute ventilation and augments the hyperventilatory response to hypoxia but not to hypercapnia (293).

On isolated CB tissue, the inhibitory impact of adenosine receptor antagonism on chemoafferent activity is more prominent in mild compared to severe hypoxia (10). ATP appears to account for the majority of the increase in chemoafferent activity in severe hypoxia. Consistent with these findings, whilst the extracellular adenosine concentration was raised in mild hypoxia, no further increase was observed in more severe hypoxic conditions (10).

The increase in extracellular adenosine concentration measured following CB exposure to mild hypoxia (10, 11) may be dependent on an up-regulation of extracellular CD39 and CD73 activity following the concurrent increase in neurosecretion of ATP from type I cells (34). ATP may also be released from sympathetic terminals as a co-transmitter in hypoxia, although no study has examined this in detail. Alternatively, augmented

intracellular adenosine generated within type I cells during hypoxia may be released into the synapse through ENT.

It has been reported that extracellular release of adenosine from the whole CB in mild hypoxia was depressed by both pharmacological inhibition of ENT and CD73 (11). Mild hypoxia induced increases in extracellular ATP were augmented 3-4 fold by CD73 inhibition indicative of an extremely high rate of extracellular ATP catabolism (10). These data are indicative of a role for both intracellular and extracellular ATP catabolism in the synaptic generation of adenosine during mild hypoxia. As yet the relative functional contribution of intracellular and extracellular derived adenosine on establishing the CB sensitivity to hypoxia has not been investigated. The effect of adenosine, produced purely from CD73 activity, in setting the stimulus threshold required for chemoafferent activation in response to mild mitochondrial inhibition and hypercapnia has not been previously evaluated. The impact of adenosine derived selectively from CD73 on modulating functional chemoafferent responses to all of these stimuli was therefore examined directly in this thesis. The potential role of synaptic adenosine derived from intracellular or extracellular ATP catabolism in mediating carotid body function is summarised in Figure 1.4.

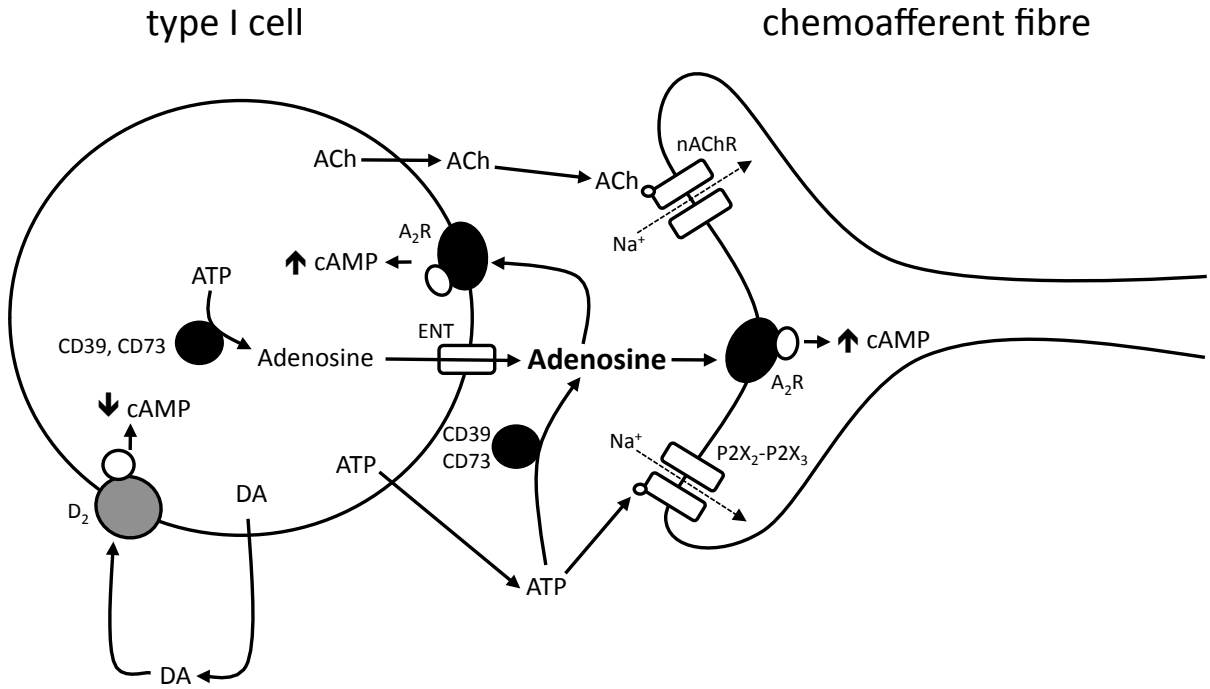


Figure 1.4 Summary of some of the neurotransmitters in the carotid body.
The type I cell contains a number of stored neurotransmitters that are released following an increase in intracellular Ca^{2+} in hypoxia or hypercapnia. Strong evidence suggests that ATP directly stimulates $P2X_2$-$P2X_3$ heteromultimers on the post-synaptic membrane and is responsible for a large component of the increase in action potential frequency in the adjacent chemoafferent fibre. ACh is also regarded as an excitatory co-transmitter that acts on post-synaptic nAChRs. In contrast, DA exerts an autoinhibitory action on type I cell function mediated through stimulation of pre-synaptic D_2 receptors and a reduction in intracellular cAMP. Adenosine is acknowledged as an excitatory neuromodulator of CB function acting on pre and post-synaptic A_2 receptors. There are two potential sources of synaptic adenosine 1) intracellular catabolism of ATP and the release of adenosine through ENT and 2) extracellular catabolism of ATP by synaptic CD39 and CD73. The contribution of these two sources of adenosine on establishing the CB sensitivity to physiological or pathological stimuli is currently not well characterised. Adapted from Nurse, 2010 (294)

1.8.5 The mechanisms accounting for excitatory modulation of the carotid body by adenosine

Extracellular adenosine produced in normoxic and hypoxic conditions has the potential to act post-synaptically on the afferent neurone or pre-synaptically on the type I cell in order to modulate CB excitability. Four G-protein coupled adenosine receptors have been cloned to data (A_1, A_{2A}, A_{2B} and A_3) and exert their actions through inhibition or excitation of adenylate cyclases and production of cAMP (reviewed in (295)).

In the CB, in situ hybridization and immunohistochemical techniques have been used to identify A_{2A} receptor mRNA and protein in type I cell clusters and in petrosal neurones (273, 296). A_{2B} receptor protein has also been detected in dissociated type I cells (297). In contrast, A_1 and A_3 receptors do not appear to be present in the CB (273, 296).

The precise functional contribution of A_{2A} and A_{2B} receptors in modulating chemoafferent activity under basal conditions and during hypoxia or hypercapnia induced chemostimulation remains unresolved. Conde et al. reported that caffeine, a non-selective A_2 receptor antagonist, inhibited ^{3}H-CA secretion and CSN discharge under basal conditions and during hypoxia, *in vitro* (297). A_{2B}, but not A_{2A}, receptor antagonists mimicked the effects of caffeine on catecholamine secretion, suggestive of a selective A_{2B} mediated action of adenosine on the type I cell. A_{2B} receptor antagonism also reduced the hypoxia stimulated increase in CSN discharge frequency. Since further inhibition of the chemoafferent activity was observed in the presence of A_{2A} antagonists it was concluded that adenosine mediated stimulation of the CB was a consequence of presynaptic A_{2B} and post-synaptic A_{2A} receptor activation (297).

Stimulation of adenosine receptors leads to changes in $[cAMP]_i$ through either activation or inhibition of transmembrane adenylate cyclases. It has been demonstrated that exogenous adenosine increased cAMP content of the whole rat CB, *in vitro* (298). In a later investigation, non-specific A_2 receptor agonists augmented whole CB cAMP concentrations in normoxia and counteracted the decrease in cAMP elicited by D_2 receptor activation (275). Since these effects were mimicked by A_{2B} but not A_{2A} agonists, it was proposed that pre-synaptic interactions between A_{2B} and D_2 receptors account for the overall $[cAMP]_i$ in the type I cell.

In contrast with the findings described above, it has been reported that an increase in type I cell $[Ca^{2+}]_i$, observed following application of adenosine, was abolished by selective A_{2A} receptor antagonism (299). The rise in $[Ca^{2+}]_i$ was mimicked by forskolin (cAMP activator), abolished by H89 (PKA inhibitor) and attenuated by anandamide (TASK channel blocker). This led the authors to promote a mechanism of adenosine mediated type I cell depolarisation dependent on pre-synaptic A_{2A} receptor stimulation, cAMP generation, PKA activation and TASK channel inhibition. However, it has also been reported that pre-synaptic A_{2A} receptor stimulation reduced the inward Ca^{2+} conductance under hypoxic conditions (296). This is consistent with an inhibitory rather than excitatory action on type I cell excitability. An explanation for these collective disparities has not been established and comprehensive characterisation of the downstream adenosinergic signalling pathways within the type I cell and the petrosal neurone leading to an increase in chemoafferent discharge remains to be more clearly defined.

1.9 The clinical implications of carotid body plasticity

The CSN chemoafferent fibres form synapses with neurones in the NTS in the medulla. The functional consequence of CB stimulation is the induction of a series of well characterised cardiovascular, respiratory and endocrine reflex responses. Specifically, these include hyperventilation, tachycardia, systemic vasoconstriction (secondary to an elevation in vascular sympathetic outflow), and an increase in adrenaline secretion from the adrenal medulla (1). Chronic up-regulation of these reflex pathways, secondary to plastic changes in CB function, is implicated in a number of clinical conditions or diseases including sleep disordered breathing (SDB), chronic heart failure (CHF) and spontaneous/essential hypertension (2). Currently, research is focusing on characterising the mechanisms underpinning CB hyperactivity in these disease states in order to develop treatments that may restrict the progression of disease morbidities and improve patient prognoses.

1.9.1 Sleep disordered breathing and chronic intermittent hypoxia

In the western world, estimates based on sample population studies suggest that the prevalence of SDB in the middle aged or elderly is 19-24% in males and 9-15% in females, (300, 301). SDB is characterised by periods of apnoea or hypopnoea (≥ 30% airflow cessation) occurring at a rate of ≥ 5 events per hour, with each being accompanied by a ≥ 4% decrease in oxyhaemoglobin saturation (302, 303). Patients with SDB are more likely to develop cardiovascular related diseases such as coronary heart disease and heart failure (304) and have increased risk of cerebral vascular

events and death (305). SDB is also associated with a resting elevation in sympathetic outflow (306, 307) and an increased incidence of hypertension (302, 308).

Proposed silencing of the CB chemoafferent activity by administration of 100% O_2 has been shown to reduce the muscle sympathetic nerve activity (MSNA) and mean arterial blood pressure (MABP) in patients with SDB, but not in healthy controls (309). This suggests that tonic input from the CB significantly contributes to the generation of the elevated MSNA and hypertension in these patients. Similar findings have been observed in animals following pre-conditioning with chronic intermittent hypoxia (CIH). Fletcher and colleagues identified that CB denervation prevented the rise in MABP in rats following 35 days of CIH (310). In a later study it was observed that the CIH induced increase in MABP was dependent on the preservation of both CB chemoafferent activity and sympathetic outflow, implying that both are up-regulated in these disease models and together are central to the emergence of hypertension.

It has been reported that patients with SDB have an augmented ventilatory response to hypoxia, combined with an amplification of hypoxia induced sympathetic outflow (311, 312). Animals pre-conditioned with CIH exhibit an elevated basal CB chemoafferent activity in normoxia (termed sensory long term facilitation; sLTF) and exaggerated chemoafferent responses to hypoxia (313, 314). Interestingly, these effects are abolished by administration of a SOD mimetic during the CIH conditioning period (313). This provides strong evidence supporting the notion that CB hypersensitivity following CIH is critically dependent on an elevation in type I cell ROS production.

The proposed mechanism leading to the tonic up-regulation of ROS generation and sLTF following CIH appears to involve a number of different pathways but ultimately relies on the increased type I cell expression and activity of NADPH oxidase 2 (NOX-2) (315). Conclusive evidence has demonstrated that sLTF following CIH cannot be induced in CBs isolated from mice that are deficient in $gp91^{phox}$, a key membrane subunit of NOX-2 (315). Furthermore, pharmacological inhibition of NOX, *in vitro*, can completely prevent any CB sLTF induced by CIH (315).

Chronic NOX-2 activation seems to be secondary to 5-HT_2 receptor stimulation. 5-HT is released in hypoxia (315) and intermittent exogenous application of 5-HT can evoke sLTF in isolated CBs *in vitro* (316). In this study, the 5-HT induced sLTF was abolished by 5-HT_2 receptor antagonists and by inhibition of PKC. In addition, the NOX-2 phosphorylation elicited by 5-HT was also attenuated by inhibitory targeting of PKC. 5-HT application also failed to induce sLTF in CBs isolated from mice that were deficient in $gp91^{phox}$. In summary, the authors proposed that intermittent release of 5-HT during CIH and subsequent 5-HT_2 receptor stimulation gave rise to chronic up-regulation in PKC activity, thus promoting NOX-2 phosphorylation, activation and ROS generation.

The relative balance of hypoxia inducible factors HIF-1α and HIF-2α expression and activity within the type I cell may also be important in conferring CB sLTF following CIH. HIF-1α does not appear to signal the acute response to hypoxia in the type I cell. However, Peng and colleagues found that, following CIH, CBs harvested from mice partially deficient in HIF-1α (HIF-$1\alpha^{-/+}$) did not exhibit any sLTF (317). In addition, augmentation of the hypoxic ventilatory response observed in wild type controls was absent in the HIF-$1\alpha^{-/+}$ mice, indicating that HIF-1α stabilisation was necessary for

promoting the elevation in CB excitability. In PC12 cells HIF-1α and NOX-2 expression is augmented following periods of CIH (318, 319). Pharmacological inhibition or siRNA knockdown of HIF-1α in PC12 cells significantly reduces the degree of NOX-2 expression (319). This illustrates a mechanism by which NOX-2 can be regulated in CIH by HIF-1α in PC12 cells, but whether the equivalent mechanism exists in the CB type I cells remains to be confirmed.

Unlike HIF-1α, HIF-2α is considered to be active under normal conditions, and exerts an inhibitory action on CB hypoxic sensitivity (320). In PC12 cells, reduced levels of HIF-2α have been detected following CIH, indicative of an increased rate of protein degradation (321). Since mice partly deficient in HIF-2α have decreased levels of *sod-2* mRNA expression (encoding the mitochondrial isoform of SOD) in CB type I cells (320), it has been suggested that following CIH, lower concentrations of HIF-2α may be coupled to increased levels of mitochondrial ROS, thereby further amplifying the CB sLTF (322).

The specific downstream targets of ROS generated from NOX-2 or the mitochondria following CIH are still to be identified. Associations between CIH, ROS generation and an inhibition of mitochondrial complex I activity have been established both in PC12 cells (323) and in CB type I cells (313). Given the strong link between mitochondrial function and CB activity, it is perhaps logical to hypothesise that the sLTF following CIH may be a consequence of impaired mitochondrial energy respiration and a change in overall cellular energy status.

1.9.2 Chronic heart failure and spontaneous hypertension

Patients with CHF present with higher levels of resting sympathetic nerve activity (SNA) and it has been reported that in moderate/severe cases, the sympathetic bursting frequency is more than 100% elevated compared with healthy age matched controls (324). In addition, these patients also exhibit an augmented ventilatory response to hypoxia (325). The direct contribution of the CB in mediating the rise in basal SNA at rest has not been evaluated in human CHF patients. However, in animals with pacing induced heart failure, chemical suppression of chemosensory activity with 100% O_2 has been shown to reduce resting renal SNA, whereas it had no effect on healthy controls (326). These CHF animals also displayed an increased chemoafferent response to hypoxia, indicative of an elevation in CB hypoxic sensitivity.

Modifications in type I cell chemoreceptor function in CHF appear to involve a number of different signalling pathways. It has been proposed that the magnification of chemoafferent activity is a consequence of a reduction in NOS-1 derived NO generation (327, 328) and down-regulation of CO production (329). Li and colleagues reported that *in vivo* application of an adenovirus containing NOS-1 DNA was able to completely restore CB NO production and reversed the CB hypoxic hypersensitivity (328). However, it has also been suggested that NOX-2 activity is up-regulated in type I cells in CHF and this in turn leads to an elevation in cellular ROS generation (330). In this instance it is thought that the increase in NOX-2 expression and activity is dependent on signalling through the angiotensin 1 receptor pathway (331) (330). The precise downstream targets of NOX-2 derived ROS are unknown. However, in a recent study, Ding and colleagues demonstrated that insertion of DNA encoding mitochondrial SOD was able to

completely restore normal CB hypoxic sensitivity in CHF animals (332). Therefore, the excitatory impact of ROS may be conferred through chronic inhibition of mitochondrial complexes and a change in the type I cell energy status. Demonstration of this mechanism in the type I cell remains to be confirmed.

Recent evidence also supports the hypothesis that the increase in sympathetic outflow and development of hypertension in spontaneous hypertensive animals is dependent on chemoafferent input from the CB (333). If similar evidence is found in humans then this could have important clinical implications for patients with essential hypertension who are known to have elevated basal levels of SNA (334). The increased excitability of the CB type I cells in spontaneous hypertensive animals may in some part be due to increased expression of TASK-1 and ASIC3 (335). However, identification of the full molecular adaptations in the type I cell in humans or animals with essential/ spontaneous hypertension is at an early stage. Comprehensive characterisation of the mechanisms underpinning the CB hyperexcitability may be important in order to develop better treatments for this disease in the future.

1.10 Overview of project aims

The precise mechanism by which the CB type I cell senses acute hypoxia is unresolved. One of the main hypotheses is that a reduction in O_2 impairs mitochondrial electron transport and the rate of ATP generation and causes a change in the cellular energy status. Therefore, the initial aim of the project is to examine the impact of mild mitochondrial inhibition on CB hypoxic sensitivity (Chapter 3). If these two stimuli act through the same chemotransduction pathway then they would be expected to interact and generate functional responses that were multiplicative rather than additive. Downstream of the mitochondria, the linking of cellular metabolic stress to type I cell depolarisation is hypothesised to be dependent on activation of the cellular energy sensor AMPK. The second aim of the project is to evaluate the hypoxic sensitivity of CBs isolated from animals deficient in Lkb-1; the essential upstream activator of AMPK (Chapter 4). This will provide more conclusive evidence supporting or rejecting the claim that Lkb1-AMPK signalling is necessary for CB hypoxia stimulus response coupling.

If CB stimulation is closely associated with cellular metabolic stress then it is logical to suggest that any stimulus capable of impairing ATP generation may cause chemoexcitation. It has been proposed that the CB is directly activated by physiological concentrations of low glucose. The functional chemoafferent response to glucose deprivation is examined in this thesis and also whether CB activation by this stimulus is dependent on a time dependent run-down of glycolysis (Chapter 5).

As well as a potential signalling molecule within the type I cell, ATP is also widely regarded as an essential neurotransmitter required for stimulation of post-synaptic

chemoafferent fibres. The release of ATP and extracellular catabolism by CD39 and CD73 may lead to tonic production of adenosine. As yet the neuromodulatory impact of this potentially significant ‘pool’ of extracellular adenosine on CB chemoafferent function has been largely overlooked. The role for extracellular adenosine derived from CD73 in establishing the CB sensitivity to a number of different stimuli is therefore investigated in the final results chapter (Chapter 6).

An elevation in chemoafferent activity at rest and an exaggerated CB response to hypoxia are closely associated with pathologies including SDB, CHF and essential/spontaneous hypertension. A reduction in CB chemoafferent activity in patients with these diseases may restrict reflex sympathetic outflow and the development of hypertension. This may limit the risk of cardiovascular complications and improve patient outcomes. The experiments performed in the current study are designed to more clearly establish whether a change in cellular energy status is central to CB stimulation. In addition, it is examined whether adenosine generated from extracellular catabolism of ATP is an important mediator of chemoafferent discharge frequency. It is anticipated that the findings presented in this thesis will advance the understanding of mammalian CB function and promote the future development of clinical interventions targeted to reduce CB chemoafferent activity in patients with SDB, CHF and essential/spontaneous hypertension.

2. Methods

2.1 Extracellular electrophysiological recordings

2.1.1 Surgical procedures and carotid body tissue isolation

All experiments and surgical procedures were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and were approved by the Biomedical Services Unit at the University of Birmingham. Tissue was isolated from adult male Wistar rats (50–200 g) (Chapters 3,5,6) or adult male C57BL/6J mice (Chapter 4). Anaesthesia was induced in an airtight induction chamber using 4% isoflurane in medical O_2 administered at a flow rate of 1.5–3 L / min. Any excess anaesthetic gas was scavenged using a Fluovac anaesthetic scavenging system (Harvard Apparatus) with a Fluosorber canister containing charcoal to adsorb the isoflurane vapour. The depth of surgical anaesthesia was considered to be sufficient only when the animal was determined to have an absent hind limb flexor withdrawal reflex in response to noxious (pressure induced) stimulation. The animal was transferred to a surgical table and surgical anaesthesia was continuously maintained by administration of 1.5–2.0% isoflurane in O_2 through a nose cone, at a flow rate of 1.5–3 L / min. Continuous monitoring of the depth of anaesthesia was ascertained by observing the rate and depth of breathing and by examining the hind limb flexor withdrawal reflex. Anaesthetic levels were adjusted accordingly in order to maintain surgical anaesthesia.

The animal was placed ventral side uppermost and the skin and superficial fascia of the neck were removed to expose the underlying tissues. The most superficial salivary glands and the sternocleidomastoid muscle were both retracted. The infrahyoid muscles, positioned superficial and immediately lateral to the trachea, were removed exposing the carotid sheath. The CCA was identified immediately lateral and parallel to the trachea in the carotid sheath, co-localised with the vagus nerve. The suprahyoid muscles, the greater horn of the hyoid bone and the hypoglossal nerve were all removed. The carotid bifurcation was identified branching into the ECA (superficial) and the ICA (deep). The CB was unable to be visualised, however, it was predicted to lie immediately deep to the occipital artery (a proximal branch of the external carotid artery) and just cranial to the carotid bifurcation. The glossopharyngeal nerve was exposed and a branch of this, the CSN, was identified crossing over the vagus nerve and coursing towards the carotid bifurcation and CB.

The CCA was clamped and then sectioned, caudal to the carotid bifurcation. A further incision was made cranial to the glossopharyngeal nerve so that the whole carotid bifurcation, the superior cervical ganglion, glossopharyngeal nerve, CSN and CB were all excised. The tissue was immediately placed in an ice-cold bicarbonate buffered extracellular Krebs solution containing, in mM: 115 NaCl, 4.5 KCl, 1.25 NaH_2PO_4, 5 Na_2SO_4, 1.3 $MgSO_4$, 24 $NaHCO_3$, 2.4 $CaCl_2$, 11 D-glucose, equilibrated with 95% O_2 and 5% CO_2. In order to minimise CB ischaemia, the tissue was removed within 30 seconds of clamping of the CCA. Animals were immediately killed by exsanguination.

Following isolation, the tissue was pinned out at points on the CCA, ECA and ICA in a small volume (approximately 0.2 ml) dissecting chamber with a Sylgard 184 base (Dow

Corning). The tissue was continuously superfused with a bicarbonate buffered extracellular Krebs solution containing, in mM: 115 NaCl, 4.5 KCl, 1.25 NaH_2PO_4, 5 Na_2SO_4, 1.3 $MgSO_4$, 24 $NaHCO_3$, 2.4 $CaCl_2$, 11 D-glucose, equilibrated with 95% O_2 and 5% CO_2. The superfusion solution was maintained at room temperature. Using a light microscope, the CB was identified just above the carotid bifurcation. The CSN was identified by its connection into the glossopharyngeal nerve. Connective tissue was removed and the superior cervical ganglion, branches of the vagus nerve and the occipital artery were all individually excised. The CSN was sectioned exposing nerve fibres and axons. To facilitate the later extracellular neuronal recordings, the whole tissue was partially digested by incubation in a bicarbonate buffered enzyme Krebs solution (0.075 mg / ml collagenase type II, 0.0025 mg / ml dispase type I; Sigma Aldrich), equilibrated with 95% O_2 and 5% CO_2, at a temperature of 37ºC, for 20–30 minutes.

2.1.2 Superfusion system

During experimentation, the CB was continuously superfused with a bicarbonate buffered extracellular Krebs solution containing, in mM: 115 NaCl, 4.5 KCl, 1.25 NaH_2PO_4, 5 Na_2SO_4, 1.3 $MgSO_4$, 24 $NaHCO_3$, 2.4 $CaCl_2$, 11 D-glucose, in a small volume (approximately 0.1 ml) recording chamber. This solution was continuously equilibrated with 5% CO_2 (unless stated otherwise) to hold the pH at approximately 7.4. All solutions were heated to 37ºC using a water bath (Grant W14, Grant Instruments). Transfer of the superfusate to the recording chamber was achieved through the use of a peristaltic pump (Miniplus 3, Gilson) and 2.5 mm internal diameter O_2 impermeable Tygon tubing (Anachem). Before entering the recording chamber, the solution was reheated to 37ºC using a self-developed in line heating system. The superfusate

temperature was continuously recorded using a thermister (Digitron Instruments) and the PO_2 was measured using a standard O_2 electrode (ISO2; World Precision Instruments). Both were positioned in the superfusion system at a point immediately before the solution entered the recording chamber. The solution was removed from the recording chamber by use of another peristaltic pump (R323, Watson Marlow), and it was either discarded or returned to the original measuring cylinder for recirculation. In experiments using exogenous $NaNO_2$, osmolality was balanced by appropriate subtraction of NaCl from the superfusate. For glucose free solutions, 11 mM mannitol was added to maintain constant osmolality.

2.1.3 Extracellular recordings of single and few-fibre chemoafferent neurones

Extracellular recordings of single or few-fibre chemoafferent activity were made from the cut end of the CSN using glass suction electrodes pulled from GC150-10 capillary glass (Harvard Apparatus). The glass electrode enclosed a silver-silver chloride wire connected to a NL100 Neurolog head stage (Digitimer). The voltage was amplified using a NeuroLog NL104 AC pre-amplifier (Digitimer), band-pass filtered between 50 Hz and 50 kHz (NeuroLog NL125; Digitimer) and amplified further with an AC amplifier (NeuroLog 105; Digitimer). Total amplification was x4000. Another silver chloride wire was placed in the recording chamber and was used as a reference electrode. The superfusate PO_2 was continuously measured using an O_2 electrode (ISO2; World Precision Instruments) and O_2 meter (OXELP; World Precision Instruments). The PO_2 and chemoafferent derived voltage were both recorded using a CED micro1401 (Cambridge Electronic Design) and visualised on a PC with Spike2 (version 7.1) software (Cambridge Electronic Design), as two individual waveforms. The chemoafferent voltage signal was sampled at 15000 Hz and the PO_2 at 100 Hz.

Offline analysis using Spike2 (version 7.1) (Cambridge Electronic Design) allowed for discrimination of electrical activity originating from a single chemoafferent fibre. Voltages detected above a certain threshold were collected and formed discrete 1.0 ms wavemarks (0.3 ms before and 0.7 ms after the original initial threshold trigger point). Using the in-built wavemark analysis in the Spike2 software (Cambridge Electronic Design), these wavemarks were then discriminated into discrete groups depending on wavemark voltage frequency, shape and amplitude. Wavemarks from each group were counted and binned into 10 second time intervals so that the action potential discharge frequency for each single chemoafferent fibre could be calculated over the course of the whole experiment.

2.1.4 Flow meter calibration

Flow meters with high precision valves (Cole Palmer Instruments) were used in order to equilibrate the superfusate with a desired gas mixture. This was important in experiments where specific PO_2 and PCO_2 values were required or in studies where the PO_2 needed to be gradually reduced in order to observe a graded hypoxic response. The scale values required to produce specific gas flow rates were provided by the manufacturer and these data were used to plot calibration curves for O_2, CO_2 and N_2 (Figure 2.1A). The overall outflow rate of the mixed gases was 160 ml / min and the total outflow pressure was 760 mmHg. To generate an outflow of a specific PO_2 and PCO_2, the corresponding individual gas flow rates were calculated as a necessary fraction of the overall total flow rate (160 ml / min), and the balance was made up with nitrogen. The calibration data was then used in order to determine the required scale values to achieve the individual calculated gas flow rates. An algorithm was established in Excel

(Microsoft), which calculated the precise scale values necessary to achieve any desired O_2 and CO_2 gas tensions. An example of the scale values required to achieve an increasing superfusate PO_2, with a fixed PCO_2 of 40 mmHg, is shown in Figure 2.1B.

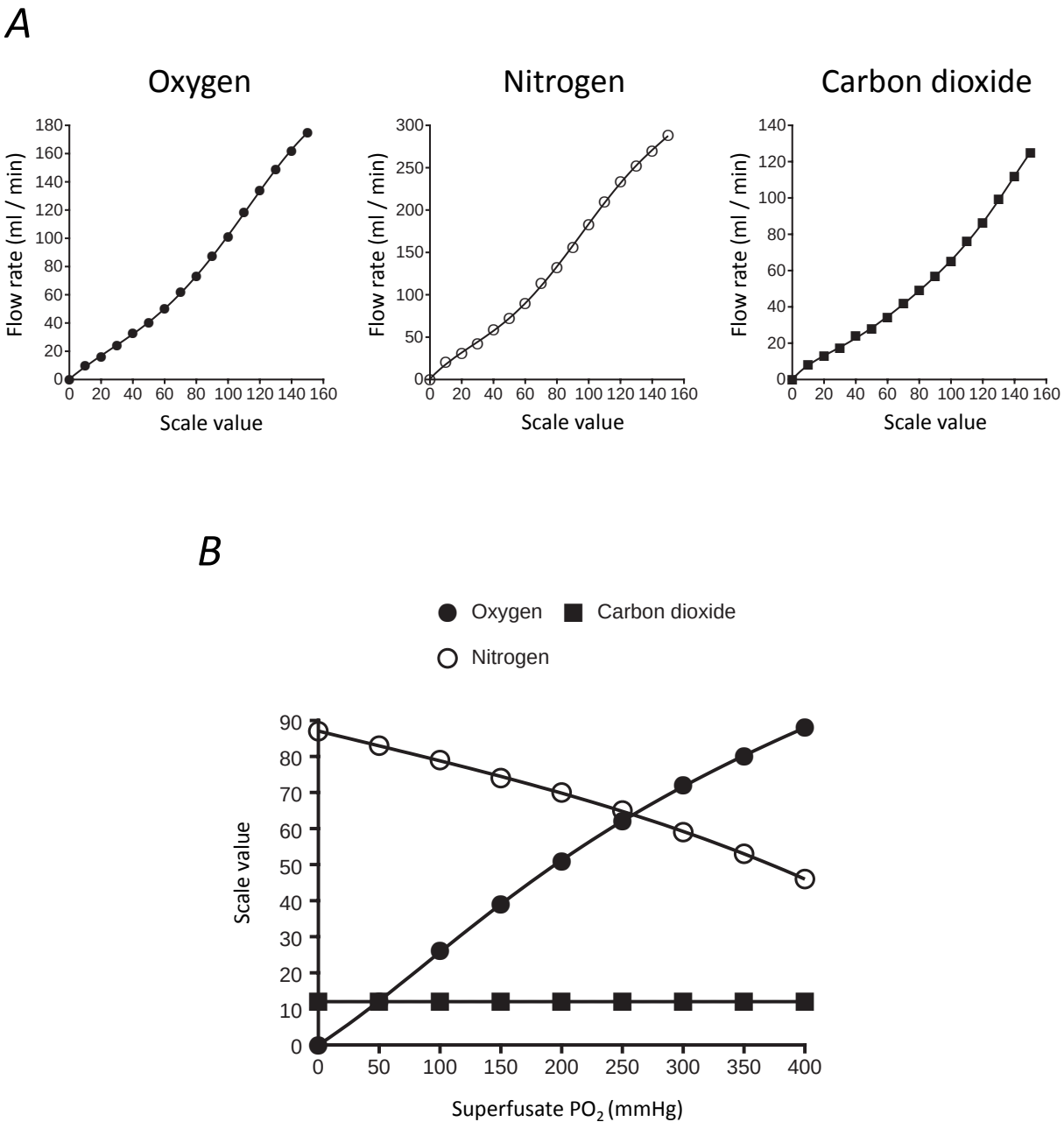


Figure 2.1 Precision flow meter calibration data used to equilibrate the superfusate with a specific PO_2 and PCO_2.
A) For each individual gas (oxygen, nitrogen and carbon dioxide) data was provided by the manufacturer (Cole-Palmer) detailing the output flow rate at given flow meter scale values. B) The superfusate could be equilibrated at different partial pressures of oxygen, nitrogen and carbon dioxide by appropriately adjusting the scale values on the flow meters. An example of the scale values required to equilibrate the superfusate with an increasing PO_2, fixed PCO_2 and decreasing PN_2 is demonstrated. The combined gas mixture outflow pressure was fixed at 760 mmHg and total flow was constant at 160 ml / min.

2.1.5 Oxygen electrode calibration

A standard O_2 electrode (ISO2; World Precision Instruments) was placed in the superfusate system, at the point of entry to the recording chamber, in order to continuously record the superfusate PO_2. The O_2 electrode signal was measured by an O_2 meter (OXELP, World Precision Instruments) and subsequently recorded by a CED micro1401 (Cambridge Electronic Design). The electrode signal was visualised as a single voltage waveform using Spike2 software (Cambridge Electronic Design). Values across a full voltage range were compared with superfusate PO_2 values, which were measured by a blood gas analyser (GEM4000; Instrumentation Laboratory). A linear relationship was calculated between the superfusate PO_2, and the corresponding recorded voltage. A PO_2 calibration curve was fitted to the equation:

$$y = mx + c$$

where y is the PO_2 in mmHg, x is the O_2 electrode recorded voltage in Volts, m is the gradient of the curve and c is the PO_2 when the recorded voltage is 0. A PO_2 calibration curve is shown in Figure 2.2. The calibration curve was used to continuously record the actual superfusate PO_2 throughout experimentation.

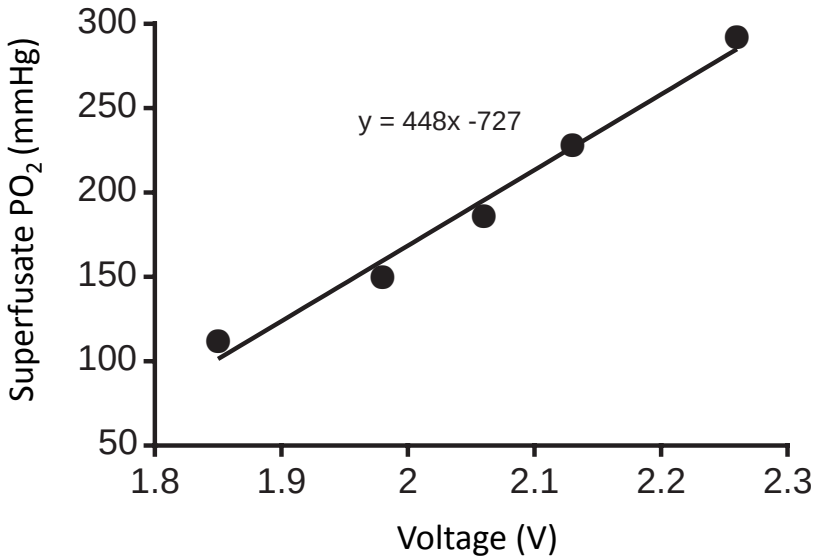


Figure 2.2 Oxygen electrode calibration curve.
Superfusate PO_2 values measured using a blood gas analyser were plotted against the corresponding voltage recorded from the O_2 electrode. Therefore an O_2 calibration curve was fitted to the equation:

$$y = mx + c$$

where y is the PO_2 in mmHg, x is the O_2 electrode recorded voltage in Volts, m is the gradient of the curve and c is the PO_2 (mmHg) when the recorded voltage is 0V. The calibration curve was used to continuously record the actual superfusate PO_2 throughout experimentation.

2.1.6 Generation of hypoxic response curves to monitor the hypoxic sensitivity of the carotid body

Functional hypoxic response curves were generated in order to directly analyse any potential changes in CB hypoxic sensitivity in the presence and absence of proposed external pharmacological or physiological stimuli, or following changes in type I cell gene expression. The single fibre chemoafferent discharge frequency was plotted against the superfusate PO_2, over a desired range of superfusate PO_2 values. To produce the hypoxic response curves, the data points were fitted to an exponential decay curve with offset, as shown below.

$$y = a + be^{-cx}$$

For the above equation, y is the single fibre discharge frequency in Hz, x is the superfusate PO_2 in mmHg, a is the discharge frequency as the PO_2 tends to infinity (offset), b is the discharge frequency when the PO_2 is 0 mmHg (minus the offset) and c is the exponential rate constant.

It has been estimated that in the *in vitro* intact rat CB preparation (approximately 200 µm diameter), the maximum difference between the superfusate PO_2 at the CB surface and the tissue PO_2 at the centre of the CB was approximately 80 mmHg (48). This was predicted as being the result of significant diffusion limitations across the whole CB tissue. Therefore, the actual PO_2 at the level of a type I cell cluster may be up to 80 mmHg lower than the recorded superfusate PO_2. It has been shown previously that functional chemoafferent hypoxic response curves observed in superfused CB preparations *in vitro* were significantly 'right shifted' compared to those generated by measuring the P_aO_2 *in vivo* (46, 48). Accordingly, in the current project, all hypoxic response curves produced from intact CB preparations were relatively 'right shifted'

compared with those obtained from *in vivo* CSN recordings (41). As a proposed consequence of the O_2 diffusion gradients across the CB tissue, hypoxic response initiation tended to be observed when the PO_2 was in the region of 125-200 mmHg. Superfusate PO_2 values of less than 100 mmHg were considered to be severely hypoxic.

As the superfusate PO_2 was gradually reduced, the single fibre chemoafferent discharge began to increase exponentially. However, if the superfusate PO_2 continued to decrease for a prolonged period, the single fibre chemoafferent frequency would reach a maximal value, plateau and then begin to diminish. For certain experiments the superfusate PO_2 was only changed to normoxia/hyperoxia after the frequency had reached a plateau or had begun to diminish in order to characterise the absolute peak frequency attained during severe hypoxia. An example of this is shown in Figure 2.3A. Importantly, if the CB was exposed to a sustained period of severe hypoxia initially, the response recorded to a second hypoxic stimulus was of a smaller magnitude (Figure 2.3A). In addition, the calculated hypoxic response curve was comparatively ‘left shifted’ suggestive of a depression in CB hypoxic sensitivity (Figure 2.3B).

In order to generate multiple reproducible hypoxic response curves the superfusate PO_2 was changed to normoxia/hyperoxia (thereby terminating the response) before a maximum chemoafferent frequency had been attained. In these instances, normoxia/hyperoxia was restored when the single fibre discharge frequency was observed as being approximately 10 Hz. An example of two reproducible hypoxic response curves produced from the same single chemoafferent fibre in the same preparation is demonstrated in Figure 2.4. The ability to generate reproducible hypoxic

response curves under control conditions was essential in order to reliably examine the impact of external pharmacological or physiological stimuli on CB hypoxic sensitivity.

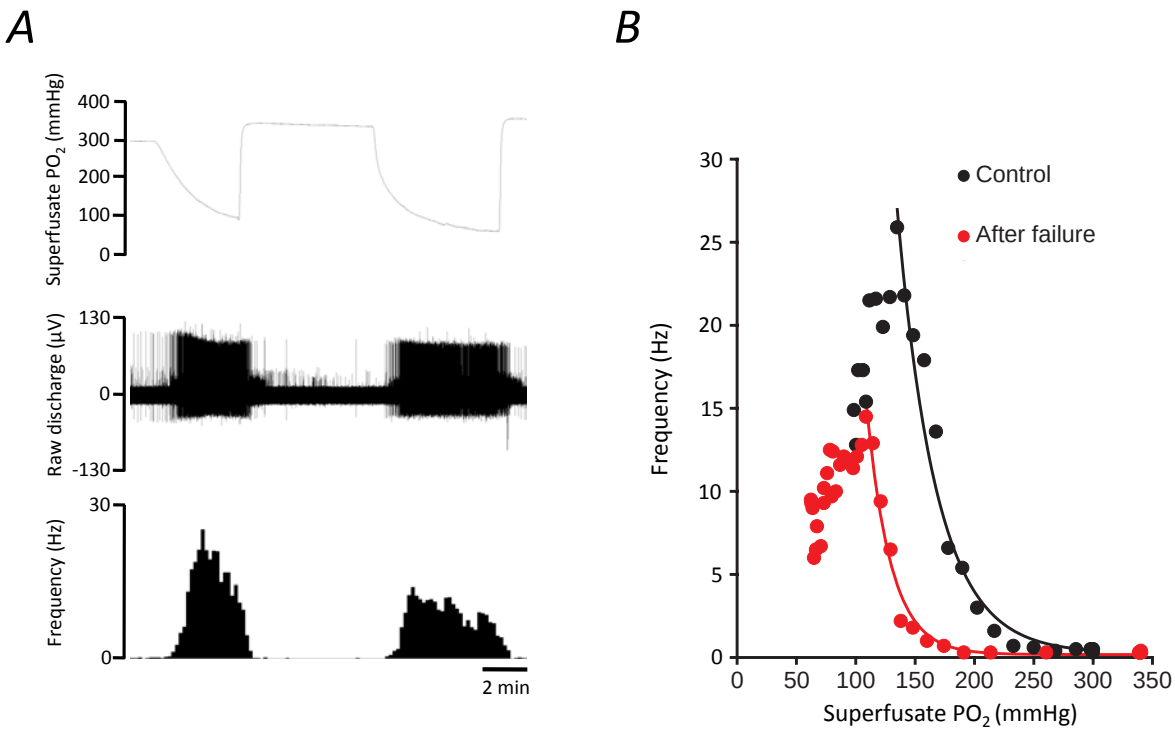


Figure 2.3 An example of two consecutive carotid body chemoafferent responses to severe hypoxia.
A) The superfusate PO_2 was continuously recorded and is shown in the upper panel. Raw neuronal discharge is demonstrated (middle) along with the single fibre frequency histograms (lower) grouped in 10 s intervals. During the initial hypoxic stimulation the chemoafferent frequency increased exponentially, but began to diminish (fail) when the hypoxic stimulus became too severe. The peak frequency observed during a secondary hypoxic stimulation was not of the same magnitude. B) Single fibre chemoafferent discharge frequency was plotted against the superfusate PO_2 during hypoxia. For generation of hypoxic response curves, the data points were fitted to an exponential decay curve with offset. For the control curve the exponential elevation in chemoafferent discharge frequency peaked and subsequently failed in more severe hypoxic conditions. The hypoxic response curve generated to a secondary hypoxic stimulus was 'left shifted' indicative of a run-down in hypoxic sensitivity.

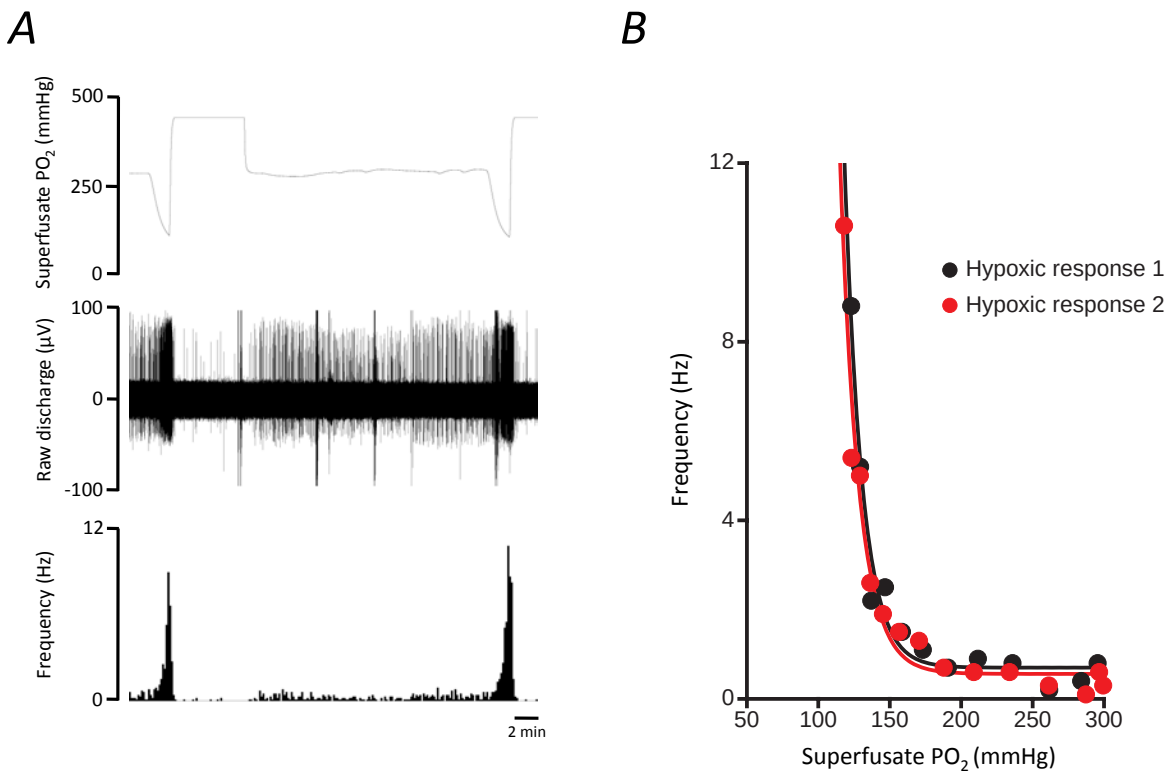


Figure 2.4 Two reproducible hypoxic response curves observed from the same single chemoafferent fibre in the same experiment.
A) The superfusate PO_2 was continuously recorded and is shown in the upper panel. Raw neuronal discharge is demonstrated (middle) along with the single fibre frequency histograms (lower) grouped in 10 s intervals. B) Single chemoafferent fibre discharge frequency was plotted against the superfusate PO_2 for the two consecutive hypoxic responses in the same experiment, separated by approximately 30 minutes. The initial response was reversed well before the peak chemoafferent frequency had been achieved to limit the possibility of reducing the sensitivity of the CB to subsequent hypoxic stimulation. Reproducibility in standard conditions was important if hypoxic sensitivity was to be later assessed in test conditions.

2.1.7 Analysing the parameters of the hypoxic response curves to identify changes in carotid body hypoxic sensitivity

Specific components of the calculated hypoxic response curves were compared to identify any potential changes in CB hypoxic sensitivity. Firstly, comparison of the exponential rate constant between two hypoxic response curves allowed for determination of any alteration in the rate of increase in chemoafferent frequency per mmHg reduction in the superfusate PO_2. Secondly, using the calculated curves, the discharge frequency could be derived from any defined level of superfusate PO_2. Therefore, the difference in discharge frequency between two different hypoxic response curves could be determined at any level of superfusate PO_2. This was important for investigating if the impact of pharmacological or physiological stimuli on CB function was PO_2/hypoxia dependent. This method of analysis is outlined in Figure 2.5A.

Thirdly, for any given discharge frequency, the corresponding PO_2 could be calculated using the inverse function of the exponential decay curve,

$$x = (Ln((y – a)/b))/-c$$

where x is the PO_2 in mmHg, y is the single fibre discharge frequency in Hz and a,b and c are constants as above. Specifically, superfusate PO_2 levels were compared when the single fibre chemoafferent discharge frequency was at 5 Hz (see Figure 2.5B). A value of 5 Hz was chosen as it lies on the exponential region of the hypoxic response curve, but is not of a magnitude at which the discharge is likely to have begun to diminish (see Figure 2.5B and 2.3B). This method was used to clearly define any PO_2 shift in the hypoxic response curve thereby providing information of a potential change in the PO_2 threshold required for hypoxic response initiation.

In specific studies using CBs isolated from transgenic mice, the single fibre discharge frequency did not always reach 5 Hz even in severe hypoxia (see Chapter 4). In these cases, a shift in the hypoxic response curve was defined by comparing the superfusate PO_2s at which the peak hypoxic frequency was achieved.

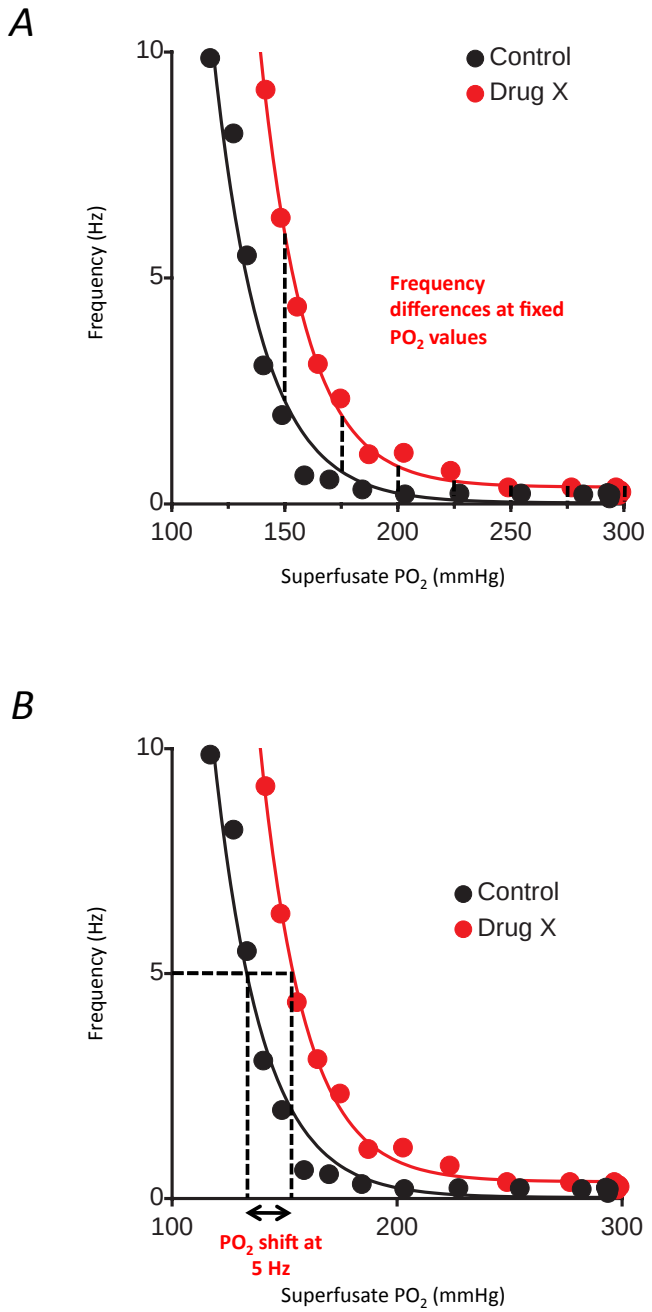


Figure 2.5 Examples of two methods used to define changes in carotid body responses to hypoxia.
A) Using the hypoxic response curve equation, the discharge frequency could be calculated at any defined level of superfusate PO_2. Therefore, the difference in discharge frequency between two different hypoxic response curves could be determined at any level of superfusate PO_2. This was important for investigating if the effect of pharmacological or physiological stimuli on CB chemoafferent discharge frequency was PO_2/hypoxia dependent. B) For any given discharge frequency the corresponding superfusate PO_2 could be calculated using the inverse function of the hypoxic response curve equation. Comparison of the PO_2 at a 5 Hz frequency was used to define any potential shift in the hypoxic response curve. A significant change in the PO_2 required to achieve a 5 Hz frequency would suggest an alteration in the PO_2 threshold required for hypoxic response initiation.

2.1.8 Monitoring the chemoafferent response to an increase in superfusate PCO_2 to assess changes in carotid body hypercapnic sensitivity

Single or few fibre CB chemoafferent responses to hypercapnia were induced by raising the superfusate PCO_2 from approximately 40 mmHg to 80 mmHg at a constant PO_2, as has been previously reported for the intact *in vitro* CB preparation (243). Since the chemoafferent response to hypercapnia is thought to peak initially and then adapt to a lower sustained frequency (187), measurements of chemoafferent activity were taken from the fifth minute of the hypercapnic stimulus (PCO_2 approximately 80 mmHg) after a relatively steady state frequency had been achieved. The elevation in single fibre frequency evoked by this level of hypercapnia was observed to be considerably less than that induced by moderate or severe hypoxia. This was consistent with previous reports identifying that the frequency response to supra-physiological concentrations of CO_2 (at normoxic/hyperoxic O_2 tensions) measured only approximately 10-50% of the peak response to severe hypoxia (46, 185, 209).

It has been observed that the single fibre chemoafferent discharge frequency increases in a linear manner over a range of approximately 25-80 mmHg P_aCO_2 (40, 41, 185). At a higher or lower P_aCO_2 the discharge frequency tends to plateau (40, 186). A similar linear relationship between superfusate PCO_2 and chemoafferent discharge has been reported for the intact CB preparation *in vitro* (46). In view of these findings it was assumed that in the present study there was a linear increase in chemoafferent activity between the two PCO_2 values (40 and 80 mmHg) at which the discharge frequency was measured. CO_2 sensitivity was subsequently estimated as the predicted increase in single fibre discharge frequency per mmHg increase in superfusate PCO_2 (ΔHz / mmHg PCO_2), given that the rise in discharge frequency was linear over the superfusate PCO_2

range used (40-80 mmHg PCO_2). This method of estimating single fibre CO_2 sensitivity was equivalent to that previously described for the intact superfused CB preparation (243).

2.2 Isolation of carotid body type I cells and detection of Ca^{2+} and NADH fluorescence

2.2.1 Carotid body type I cell dissociation

All experiments using dissociated CB type I cells were performed at the University of Oxford in collaboration with Dr K. Buckler. CBs were harvested from 9–12 day old immature male rats. The method of surgical isolation was as described in Section 2.1.1 except that halothane was used as the inhalation anaesthetic. In accordance with Home Office legislation, all animal handling and surgical procedures were performed by Dr K. Buckler and not by the author of this thesis.

The whole carotid bifurcation, the superior cervical ganglion, CSN, and CB were all excised together and immediately placed in a dissecting dish containing an ice-cold bicarbonate buffered PBS solution (Dulbecco's PBS; Sigma-Aldrich) pre-equilibrated with 95% O_2, 5% CO_2. The tissue was pinned out at points on the CCA, the ICA and ECA with the SCG upper most. The SCG was removed along with the vagus nerve. In this orientation the CB was located superficial to the occipital artery near to the carotid bifurcation. Any fat or loose connective tissue was removed. The CB was teased away from the adjacent arterial walls and then placed in another ice-cold bicarbonate buffered PBS solution (Dulbecco's PBS; Sigma-Aldrich) pre-equilibrated with 95% O_2, 5% CO_2.

After both CBs had been dissected out they were transferred into a bicarbonate buffered enzyme solution containing 2 ml HAMS F-12 nutrient mixture (Sigma-Aldrich), L-glutamine (2 mM) (Sigma-Aldrich), penicillin (100 IU / ml) (Sigma-Aldrich), streptomycin (100 µg / ml) (Sigma-Aldrich), insulin (4 µg / ml) (Sigma-Aldrich), collagenase (0.4 mg /

ml) (Sigma-Aldrich) and trypsin (0.2 mg / ml) (Sigma-Aldrich). The CBs and enzyme solution were placed in a tissue culture incubator at 37°C, 5% CO_2 and 21% O_2, for 23 minutes. CBs were then removed from the incubator and gently teased apart before being placed back into the incubator for a further 7 minutes.

CBs were subsequently transferred to an enzyme free bicarbonate buffered solution containing 1 ml HAMS F-12 nutrient mixture (Sigma-Aldrich), L-glutamine (2 mM) (Sigma-Aldrich), penicillin (100 IU / ml), streptomycin (100 µg / ml) (Sigma-Aldrich), insulin (4 µg / ml), FBS (10% v/v) (Sigma-Aldrich) and trypsin inhibitor (0.5 mg / ml) (Sigma-Aldrich), for 4 minutes. This was the standard culture medium used. CBs were transferred to a clear fluorimeter cuvette (Sigma-Aldrich) containing the same culture medium. CBs were triturated using fine bore glass pipettes (200, 100 and 50 µm internal diameter), which had previously been heat-sterilised. 30 µl aliquots of the cell suspension were then transferred to individual poly-D-lysine coated 6 mm diameter coverslips (VWR). These coverslips, positioned in a culture media dish, were placed in the incubator at 37°C, 5% CO_2 and 21% O_2, for 2 hours. Afterwards, a further 2 ml of the above culture media was added to the culture dish and the dissociated type I cells were then left in the incubator until required.

2.2.2 Measurement of NADH and $[Ca^{2+}]_i$

Ca^{2+} and NADH fluorescence measurements were made using an inverted microscope (Nikon Diaphot 200; Nikon) equipped with a 100 W xenon lamp that provided the fluorescence excitation light source. Photomultiplier tubes (PMT; Thorn EMI), cooled to minus 20°C, were used to detect the emitted fluorescence. The output signal was fed through a current-voltage converter and the voltage was recorded using a CED

micro1401 (Cambridge Electronic Design) and visualised on a PC with Spike2 (version 7.1) software (Cambridge Electronic Design) as an individual waveform. The voltage signal was sampled at 250 Hz.

NADH autofluorescence was excited at 340 nm and the emission was measured at 450 ± 30 nm. $[Ca^{2+}]_i$ was determined by using the fluorescent dye, Indo-1 (336). Cells were initially loaded by incubation with the Indo-1 acetoxymethyl ester (Indo-1-AM; Sigma-Aldrich) for 1 hour. After transferring the cells to the recording chamber, cells were illuminated at 340 nm and emission was measured at 405 ± 16 nm (F_{405}; Ca^{2+} bound Indo-1) and 495 ± 10 nm (F_{495}; Ca^{2+} free Indo-1). A further channel was created using the Spike2 (version 7.1) software (Cambridge Electronic Design) that calculated and recorded the ratio of F_{405} / F_{495} (R).

In order to directly quantify the type I cell $[Ca^{2+}]_i$ the constants R_{min}, R_{max} and $F_{495\ (max\ /\ min)}$ were initially determined. To do this, type I cells loaded with Indo-1 were transferred into a HEPES buffered Krebs Ca^{2+} free solution (see below) containing 10 mM EGTA (Sigma-Aldrich) for 1 hour at room temperature. This method was performed to completely de-saturate the Indo-1. Cells were subsequently placed in a recording chamber and superfused with the same solution. Under these conditions, fluorescence measurements were made to detect the R_{min} from a single type I cell. To identify the R_{max}, the superfusate was modified to a similar HEPES buffered Krebs solution containing 2.5 mM Ca^{2+} and 10 µM ionomycin. Using this method the $F_{495\ (max\ /\ min)}$ could also be calculated. The K_d (dissociation constant) of the equation:

$$[Ca^{2+}\text{-Indo-1}] \Leftrightarrow [Ca^{2+}] + [\text{Indo-1}]$$

was taken as 250 nM as has been characterised previously (336). The $[Ca^{2+}]_i$ was calculated throughout experimentation according to the equation described by Grynkiewicz and colleagues, shown below.

$$[Ca^{2+}]_i = K_d(((R - R_{min}) / (R_{max} - R)) / (F_{495\ (max\ /\ min)})).$$

2.2.3 Solutions

The dissociated type I cells were continuously superfused with a standard bicarbonate buffered Krebs solution containing, in mM: 115 NaCl, 4.5 KCl, 1.25 NaH_2PO_4, 5 Na_2SO_4, 1.3 $MgSO_4$, 24 $NaHCO_3$, 2.4 $CaCl_2$ and 11 D-glucose. Normoxic/hyperoxic solutions were equilibrated with 5% CO_2 and 95% air. Mild hypoxic solutions were equilibrated with 5% CO_2, 2% O_2 and 93% N_2 and severe hypoxic solutions with 5% CO_2 and 95% N_2. All solutions were heated to 37ºC using a water bath (Grant W14, Grant Instruments). Transfer of the superfusate to the recording chamber was achieved through gravity via 3 mm internal diameter O_2 impermeable stainless steel tubing. In experiments using exogenous $NaNO_2$, osmolality was balanced by appropriate subtraction of NaCl from the superfusate. For glucose free solutions, 11 mM mannitol was substituted in place of glucose to maintain constant osmolality.

The HEPES buffered Krebs solution used for Indo-1 calibrations contained in mM: 140 NaCl, 4.5 KCl, 1 $MgCl_2$, 2.4 $CaCl_2$, 11 D-glucose and 20 HEPES. The Ca^{2+} free solution was deficient in $CaCl_2$ and contained 10 mM EGTA. During Indo-1 calibration procedures the HEPES buffered Krebs solution was equilibrated with 5% CO_2 and 95% air.

2.3 Carotid body tissue immunohistochemistry

The whole carotid bifurcation, the superior cervical ganglion, glossopharyngeal nerve, CSN, and CB were all surgically isolated from anaesthetised (isoflurane 1.5-2%) adult (50-200 g) male Wistar rats as described in Section 2.1.1. Following tissue procurement, all animals were immediately killed by exsanguination. The tissue was immediately fixed for 2 hours at room temperature in 2.2% formaldehyde (TAAB Laboratories) in 10 mM PBS, pH 7.4, containing 2% glucose and 0.02% sodium azide (Sigma-Aldrich). The tissue was rinsed once in PBS and left in a 30% sucrose solution (for cryoprotection) overnight before being embedded in frozen optimal cutting temperature compound (OCT; TAAB Laboratories). 10µm thick tissue sections were cut using a cryostat and adhered onto charged glass slides (Thermo Scientific). The tissue sections were washed three times in PBS, and then permeabilised by incubation in PBS containing 1% Triton X-100 (Sigma-Aldrich) for 10 minutes at 21°C. To limit non-specific antibody staining, tissue samples were incubated with PBS containing 1% BSA and 0.05% Tween20 (Sigma-Aldrich) for 30 minutes at 21°C.

The tissue sections were incubated in a 0.1% BSA, 0.1% Tween20 PBS solution containing the appropriate primary antibodies, in a humidified chamber at 4°C for 24 hours. Mouse monoclonal anti-rat tyrosine hydroxylase antibodies (Abcam) were applied (1:20) for the positive identification of type I cells. In addition, rabbit monoclonal anti-rat glycogen synthase I (1:50; Abcam) or rabbit polyclonal anti-human glycogen phosphorylase BB (1:100; AbD Serotec) antibodies were applied to establish the presence or absence of enzymes capable of glycogen synthesis and metabolism. The

rabbit polyclonal anti-human glycogen phosphorylase BB antibody was predicted to react with the rat glycogen phosphorylase BB isoform because of strong (greater than 95%) sequence homology between the human and rat isoforms (UniProt protein database).

Tissue sections were washed in a 0.1% Tween20 PBS solution to remove excess primary antibodies and then incubated in a 0.1% BSA, 0.1% Tween20 PBS solution containing anti-rabbit Alexa flura 488 (green) and anti-mouse Alexa flura 594 (red) (1:250; Molecular Probes) conjugated secondary antibodies to allow for subsequent fluorescent detection of the protein-immunoglobulin complexes. Tissue sections were washed in a 0.1% Tween20 PBS solution to remove unbound secondary antibody and then mounted in Vecta-mounting medium containing the nuclear stain DAPI (Vector Labs) (Figure 2.6). Images were viewed using an epifluorescent microscope (Zeiss).

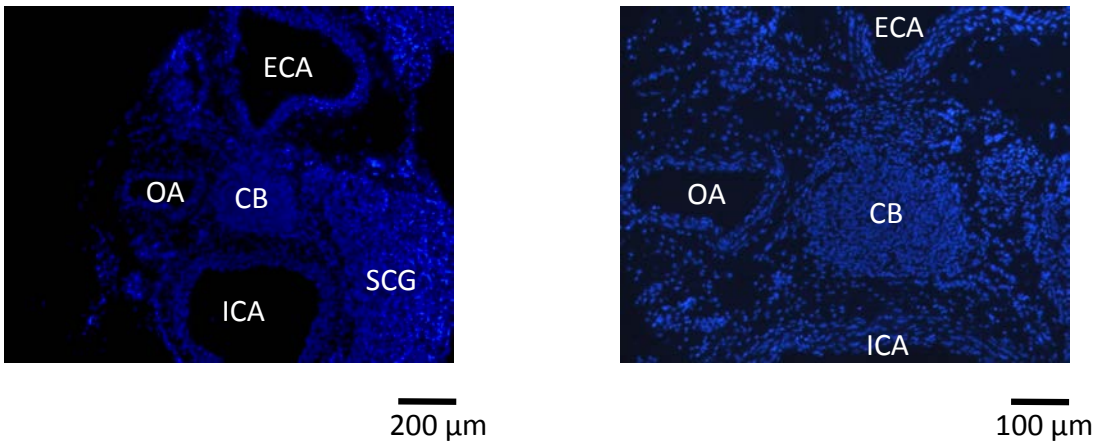


Figure 2.6 Example images showing the location of the carotid body in relation to the surrounding structures near the carotid bifurcation.
10 µm thick tissue sections were cut and stained with DAPI to specifically identify cell nuclei. In the rat, the carotid body (CB) is closely associated with the internal carotid artery (ICA), the external carotid artery (ECA), the occipital artery (OA) and the superior cervical ganglion (SCG).

3. Examining the interaction between mild mitochondrial inhibition and hypoxia in the carotid body using exogenous nitrite

3.1 Chapter introduction and overview

A unique feature of the mammalian CB is the ability to detect and respond to a fall in tissue PO_2 at levels significantly higher than those that impair the metabolism of most other cell types. Whilst a number of putative mechanisms sensitive to changes in PO_2 have been described, it is less apparent how these might contribute to this heightened O_2 sensitivity in the CB. It has been hypothesised that the hypoxia detecting mechanism may be purely a consequence of a run-down in mitochondrial electron transport and a change in the cellular energy status (4, 50, 51, 54). In support of this mitochondrial hypothesis, it has been identified that chemostimulation evoked by a number of different mitochondrial poisons shares many of the same downstream transduction processes that are known to be present in hypoxia stimulus excitation coupling. These include deactivation of background K^+ (TASK-like and TREK-1) currents (29, 54), Ca^{2+} influx through L-type Ca^{2+} channels (54), neurosecretion (52) and stimulation of the CSN afferents (50-52, 337).

For the mitochondrial hypothesis to be further strengthened, administration of mitochondrial inhibitors at sub-saturating concentrations would ideally show a degree of response interaction with hypoxia, thus evidencing that these stimuli are intimately

associated. To date, specific investigations examining the potential alterations of the CB hypoxic sensitivity caused by mild mitochondrial inhibition have not been performed.

The rapidly reversible and O_2 dependent reduction in mitochondrial energy metabolism caused by binding of NO to the haem a_3/Cu_B binuclear centre in cytochrome c oxidase is well established (177, 178). However, potential modulation of mitochondrial function by NO has not been studied directly in the type I cell. This is possibly due to the overall impact of NO on CB chemostimulation being inhibitory rather than excitatory (136, 338). This attenuation is thought to be mediated primarily by S-nitrosation and deactivation of the L-type Ca^{2+} channels and by cGMP dependent activation of BK_{Ca} current (148, 149).

Within the CB type I cell the only NOS isoform identified to date has been NOS-3 (83). The same study showed that hypoxia increased the NO content of the mitochondrial membranes. In addition, it is recognised that the NOS-3 KO mouse has an impaired ventilatory response to hypoxia coupled with a down-regulation in CB function (144). Therefore, NO produced locally at the level of the mitochondria may have excitatory actions on CB hypoxic sensitivity through direct modulation of mitochondrial function. Importantly, the impact of NO on mitochondrial activity may have been masked in previous studies using exogenous NO donors due to the additional inhibitory effect of NO in other cellular compartments.

Relatively recent findings have identified NO_2^- as a novel NO producing molecule. Generation of NO is achieved through single electron reduction of NO_2^- and is favoured in the presence of a reduced environment and under conditions of acidosis or hypoxia. Multiple enzymes have been proposed as putative NO_2^- reductases (see Chapter 1) with

the best characterised being the deoxygenated forms of the family of haem-globins (180). In the absence of a high level of cytoplasmic NO_2^- reductase activity it has also been demonstrated that the mitochondria can directly reduce NO_2^- to form NO, although this requires supraphysiological (mM) concentrations of exogenous NO_2^- (172-174). As is the case with the haem-globins, NO production from NO_2^- by the mitochondria is potentiated in an acidic and hypoxic environment (172-174). Potentially, the addition of exogenous NO_2^- may generate NO locally at the mitochondria and modify CB mitochondrial activity without directly impacting on the ion channel function in other cellular compartments.

The aims of the investigations in this chapter are summarised below:

1. To investigate if supraphysiological (mM) concentrations of exogenous NO_2^- can evoke reversible and dose dependent mitochondrial inhibition that is directly associated with CB chemoexcitation.

2. To examine if mild mitochondrial inhibition with NO_2^- subsequently changes the hypoxic sensitivity of the CB.

3. To explore if further similarities exist between mitochondrial inhibition and hypoxia, including the potential activation of AMPK and synergy with hypercapnia.

3.2 Results

3.2.1 Nitrite causes dose dependent carotid body chemostimulation that is coupled with an inhibition of mitochondrial electron transport

Experiments were initially performed on the isolated intact CB to measure the single fibre chemoafferent discharge frequency in normoxia/hyperoxia and normocapnia (superfusate PO_2 approximately 300 mmHg, PCO_2 approximately 40 mmHg). Only supraphysiological (mM) concentrations of NO_2^- elevated the chemoafferent frequency under these conditions, consistent with those known to generate NO at the mitochondria (Figure 3.1A). Chemostimulation induced by NO_2^- was rapidly induced (less than 1 minute) and well maintained throughout the application and was reversed within 1 to 2 minutes of removal from the superfusate (Figure 3.1A). Significant dose dependent increases in mean chemoafferent activity were observed at concentrations of 3.3, 10 and 33 mM NO_2^- with the mean response to 33 mM NO_2^- measuring 69.3 ± 6.5 % of the absolute peak frequency response to hypoxia (PO_2 approximately 60 mmHg) (Figure 3.1B).

The novel ability of supraphysiological (mM) concentrations of exogenous NO_2^- to induce dose dependent chemoexcitation was consistent with the hypothesis that reduction of NO_2^- to form NO subsequently inhibited type I cell mitochondrial function and activated downstream signalling pathways. To test this mitochondrial hypothesis directly, studies were subsequently performed on dissociated type I cell clusters and records of NADH autofluorescence were taken as a measure of mitochondrial function as has been previously described for the CB (4, 54). For these experiments, the superfusate was equilibrated with 2% O_2. This is the highest dissolved O_2 concentration

known to allow for NO to be generated from NO_2^- by isolated mitochondria (172) and is a level of hypoxia that causes partial, but not full, type I cell mitochondrial inhibition.

Similar supraphysiological concentrations of NO_2^- to those that increased chemoafferent frequency in the intact CB organ also generated significant elevations in NADH autofluorescence, indicative of an attenuation of mitochondrial electron transport (Figure 3.2A). Increases in NADH autofluorescence were almost instantaneous upon NO_2^- application and reversed within 1 to 2 minutes of removal from the superfusion (Figure 3.2A). The grouped data showed that addition of 10 mM NO_2^- evoked a 25.5 ± 1.3 % and 33 mM NO_2^- a 79.2 ± 13.8 % rise in NADH autofluorescence, when measured as the proportion of the mean paired peak responses to 0% O_2 (Figure 3.2B). Collectively, these data suggest that concentrations of NO_2^- capable of inducing whole organ chemoexcitation also produce concurrent impairment of type I cell mitochondrial function.

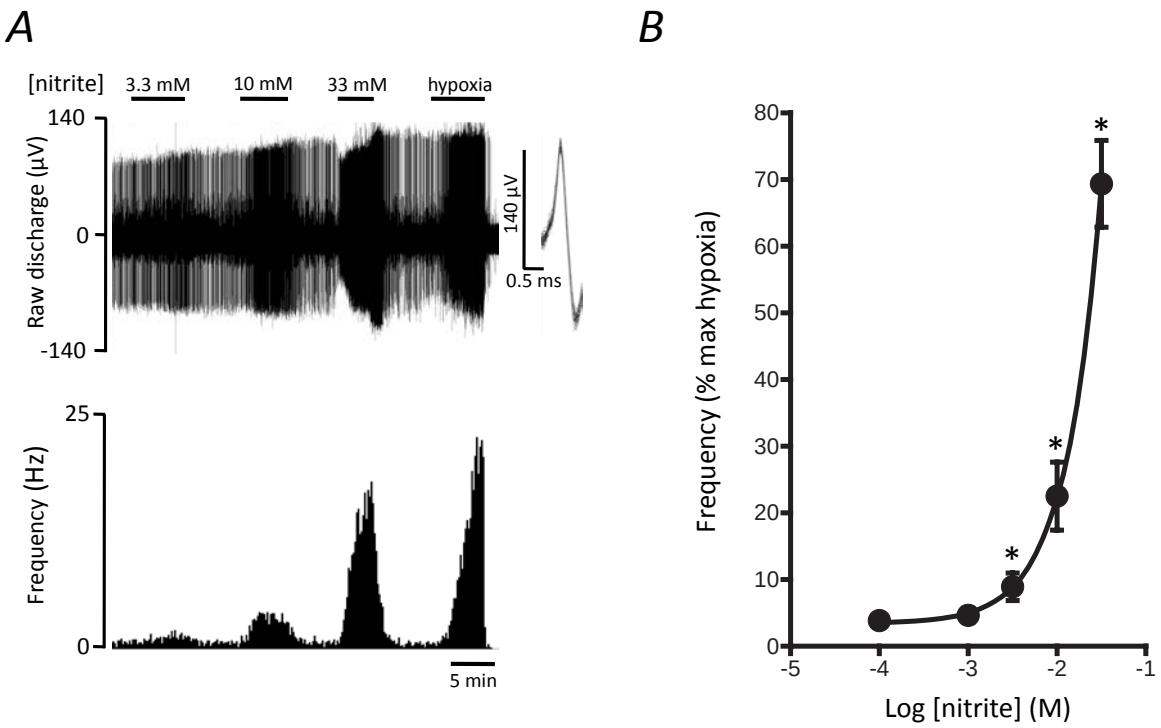


Figure 3.1 Supraphysiological concentrations of exogenous nitrite evoke increases in carotid body chemoafferent activity.
The data presented was taken from 9 fibres from 6 CB preparations. A) An example trace showing the effect of different doses of nitrite (NO_2^-) on the chemoafferent frequency recorded from a single fibre. For comparison with the peak chemoafferent response to hypoxia, the tissue was stimulated by a severe hypoxic stimulus (PO_2 approximately 60 mmHg) following the final dose of NO_2^-. Raw discharge is shown (upper) along with frequency histograms (lower) grouped in 10 s intervals. Overdrawn action potentials are shown inset to demonstrate the single fibre discrimination from which the frequency was taken.
B) Mean frequencies induced by different concentrations of NO_2^-, expressed as a percentage of the paired peak frequency response to severe hypoxia (PO_2 approximately 60 mmHg). Error bars indicate ± S.E.M.
* denotes $P < 0.05$ compared with initial NO_2^- dose; one way repeated measures ANOVA with Dunnett's post hoc analysis.

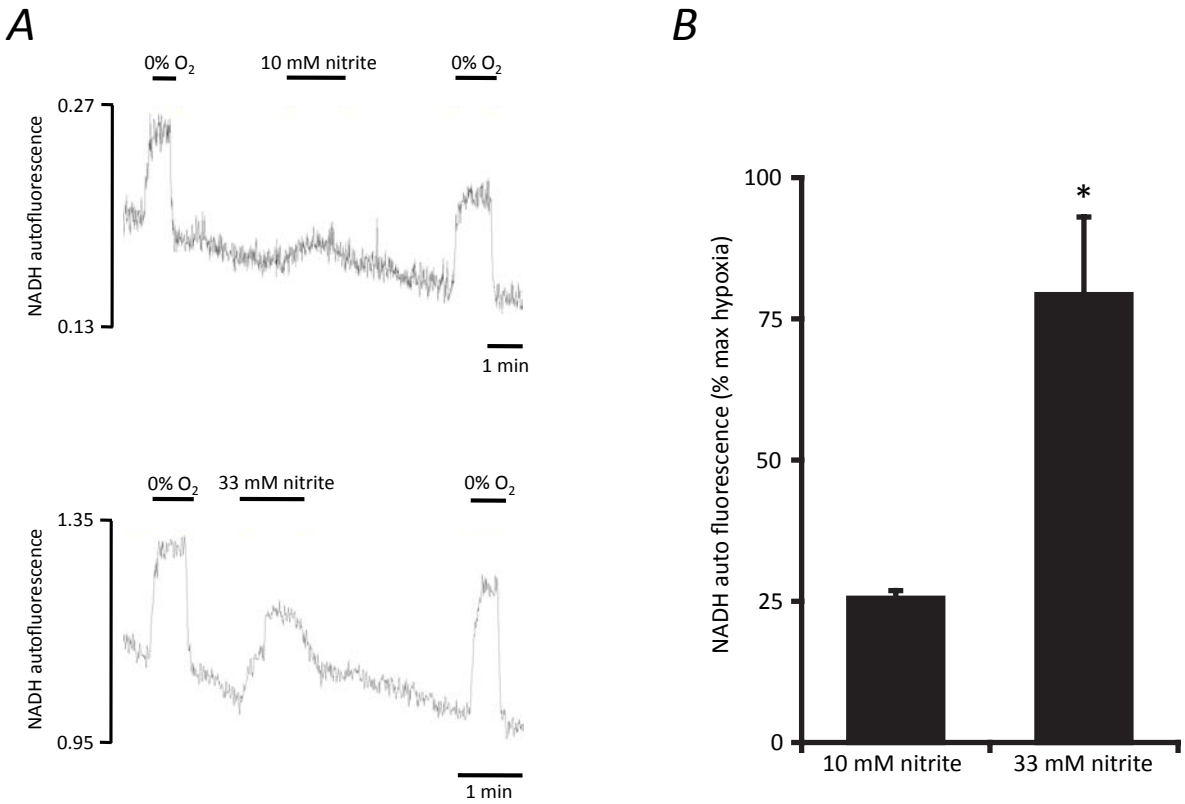


Figure 3.2 Supraphysiological concentrations of exogenous nitrite evoke increases in NADH autofluorescence indicative of a reduction in mitochondrial energy metabolism.
A) Example spectra of the effect of 10 mM and 33 mM nitrite (NO_2^-) on the NADH autofluorescence measured from two different dissociated type I cell clusters. For comparison with the change in NADH autofluorescence induced by severe hypoxia, a 0% O_2 stimulus was applied before and after addition of NO_2^-. B) Mean NADH autofluorescence induced by 10 mM NO_2^- (4 clusters, 4 CB preparations) and by 33 mM NO_2^- (6 clusters from 4 CB preparations), expressed as a percentage of the paired mean peak NADH autofluorescence responses evoked by severe hypoxia (0% O_2). Error bars indicate + S.E.M. * denotes $P < 0.05$ compared with 10 mM NO_2^-; unpaired t-test.

3.2.2 Carotid body type I cell Ca^{2+} responses to nitrite are PO_2 dependent

The binding of NO to cytochrome c oxidase is rapidly reversible and highly dependent on PO_2 with increased NO dependent mitochondrial inhibition taking place under more hypoxic conditions (179). Furthermore, mitochondrial reduction of NO_2^- to release NO is tightly regulated by PO_2 and is favoured under conditions of increasing hypoxia (172-174). Studies were undertaken to investigate whether the impact of NO_2^- on type I cell stimulation was PO_2 dependent and therefore indicative of a downstream NO mediated effect.

In these experiments, dissociated type I cell Ca^{2+} fluorescence was recorded as a measure of the level of chemostimulation intensity. Ca^{2+} responses to 33 mM NO_2^- were evaluated at two levels of dissolved O_2: 20% and 2%. An example trace from a single cluster is shown in Figure 3.3A. In 20% dissolved O_2, addition of 33 mM NO_2^- did not evoke further elevations in type I cell $[Ca^{2+}]_i$ (Figure 3.3A and B). In contrast, when the superfusion was switched to 2% O_2 (generating a small initial rise in $[Ca^{2+}]_i$), 33 mM NO_2^- stimulated an almost instantaneous and substantial rise in $[Ca^{2+}]_i$ that was well maintained and rapidly reversible (Figure 3.3A and B). Therefore, the response to 33 mM NO_2^- appeared to be critically dependent on the O_2 concentration. Since the excitatory actions of NO_2^- were only observed in hypoxia these data supported the hypothesis that the NO_2^- induced stimulation was due to the initial formation of NO.

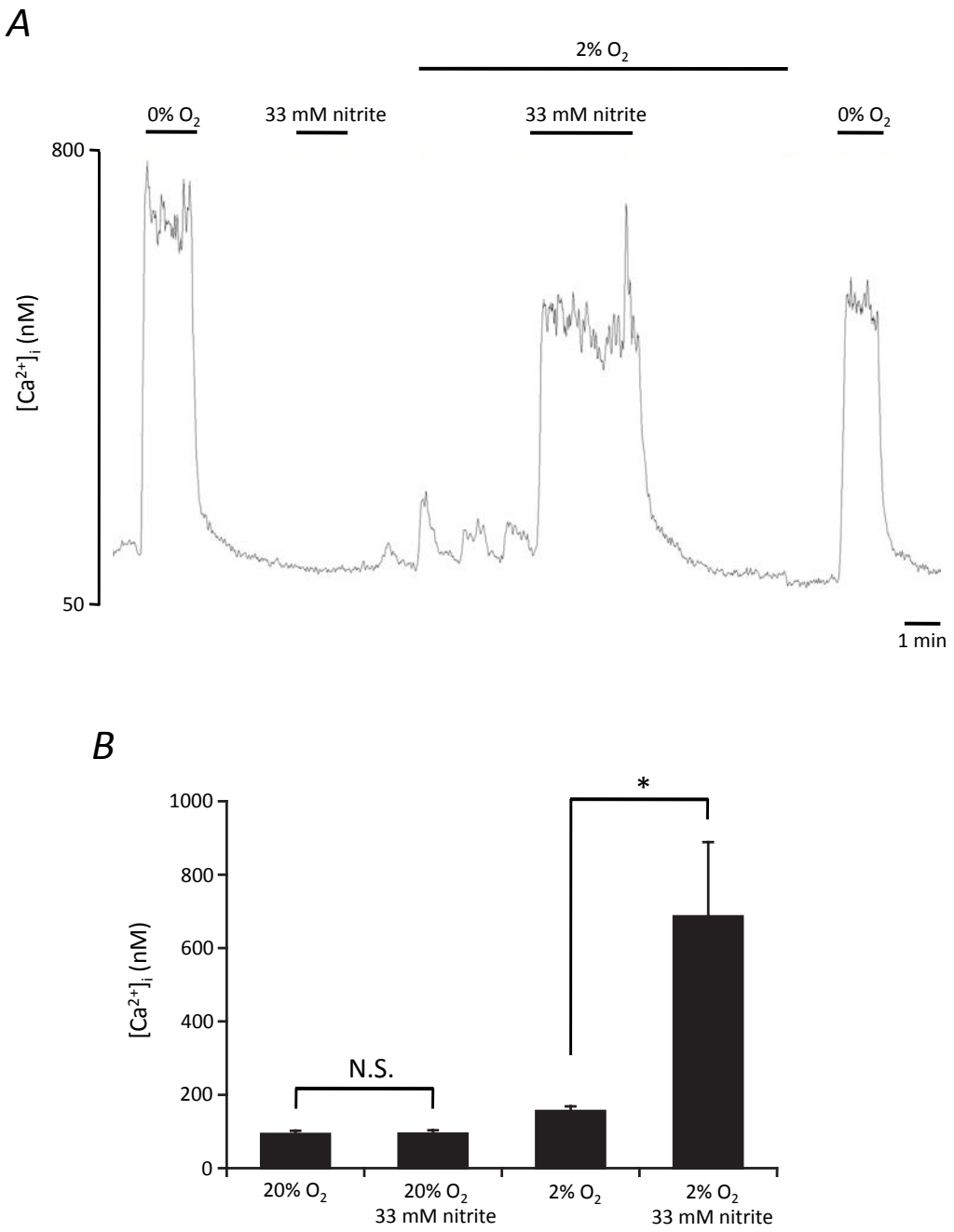


Figure 3.3 The carotid body type I cell $[Ca^{2+}]_i$ response to nitrite is PO_2 dependent.
A) Example Ca^{2+} fluorescence spectra measured from a single type I cell cluster showing the effect of 33 mM nitrite (NO_2^-) in 20% and 2% dissolved O_2. B) Mean Ca^{2+} fluorescence data taken from 3 type I cell clusters from 2 CB preparations. Error bars indicate + S.E.M. * denotes $P < 0.05$ compared with 2% O_2; one way repeated measures ANOVA with Bonferroni post hoc analysis test.

3.2.3 Mild mitochondrial inhibition with nitrite alters the carotid body response to hypoxia

Previous experiments in this chapter characterised NO_2^- as a novel and highly reversible type I cell mitochondrial inhibitor that was also able to elicit dose dependent increases in CB chemostimulation and single fibre CSN afferent activity. The ability for mitochondrial inhibitors to evoke CB stimulation is well known. However, if hypoxic sensing is purely a consequence of reduced mitochondrial energy respiration, it would be predicted that mild mitochondrial inhibition would augment the CB sensitivity to hypoxia, thereby showing that these stimuli were closely related and were not acting independently.

Experiments in this section were performed on the superfused intact CB and recordings of single fibre chemoafferent activity were made in order to generate functional hypoxic response curves. The CB was stimulated by hypoxia in the presence and absence of 3.3 mM NO_2^-, a concentration previously shown to evoke only a very small increase in basal activity (0.60 ±0.10 Hz; control, compared with 1.11 ± 0.21 Hz; 3.3 mM NO_2^-) and was hypothesised as inducing only a very mild level of mitochondrial inhibition (see 3.2.1 and 3.2.2). Importantly, the addition of 3.3 mM NO_2^- evoked a marked ‘right shift’ of the CB hypoxic response curve (Figure 3.4A). The mean ‘right shift’ was quantified by measuring the PO_2 when the discharge frequency was at 5 Hz (see Section 2.1.7). To attain a frequency of 5 Hz, the mean superfusate PO_2 was 105 ± 7 mmHg under control conditions and 141 ± 11 mmHg in the presence of 3.3 mM NO_2^- (Figure 3.4B). This effect was consistent across all fibres recorded from (Figure 3.4B). In addition, a similar ‘right shift’ was observed in the presence of 3.3 mM NO_2^- when the discharge frequency was collated from a nerve trunk preparation containing multiple fibres (Supplementary figure 1).

The rate of chemoafferent increase following hypoxic response initiation was not different in the presence of 3.3 mM NO_2^-. This was evidenced by paired exponential rate constants not being significantly different between groups; 0.032 ± 0.004 for the control and 0.032 ± 0.005 for 3.3 mM NO_2^-. Therefore, the data suggested that the overall 'right shift' of the hypoxic response curve in the presence of NO_2^- was a consequence of a change in the 'set point' or 'threshold' for hypoxia response initiation rather than a change in the rate of increase.

Analysis of the paired differences in discharge frequency calculated at fixed levels of PO_2, in the presence or absence of 3.3 mM NO_2^-, showed that the augmentation in frequency evoked by 3.3 mM NO_2^- was magnified in more hypoxic conditions (Figure 3.4A). Specifically, the excitatory impact of NO_2^- on the chemoafferent activity was enhanced at superfusate PO_2s of 125 and 100 mmHg (Figure 3.4A). These data are strongly indicative of a degree of significant multiplicative interaction between NO_2^- and hypoxic stimuli suggesting that the two stimuli act through the same pathway and are not independent. In view of the findings earlier in this chapter, it is proposed that the interaction takes place at the mitochondria and that pre-conditioning by NO_2^- (through NO production), makes the mitochondrial electron transport more susceptible to a fall in PO_2 and the CB more sensitive to hypoxia.

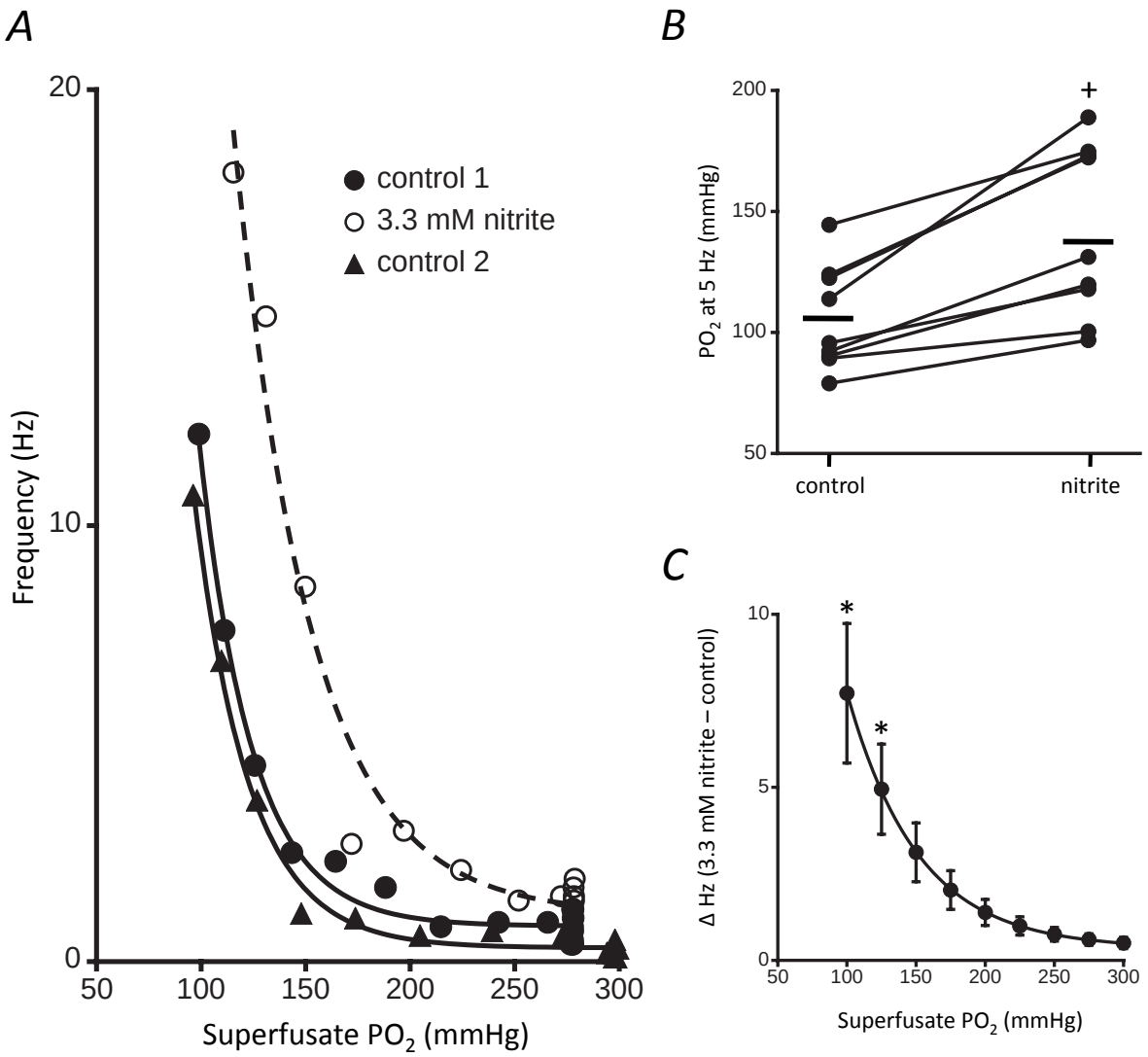


Figure 3.4 Nitrite sensitises the carotid body to hypoxia.
The data presented was from 9 fibres from 7 CB preparations A) Characteristic example from a single chemoafferent fibre demonstrating the reversible 'right shift' induced by 3.3 mM nitrite (NO_2^-) on the hypoxic response curve. B) For all fibres the PO_2 was measured at 5 Hz in the presence and absence of 3.3 mM NO_2^- in order to quantify the mean 'right shift'. + denotes $P < 0.05$, 3.3 mM NO_2^- compared with control; paired t-test. C) The calculated augmentation in single fibre discharge frequency induced by 3.3 mM NO_2^- was plotted over a range of defined superfusate PO_2 values and shows an enhanced impact in hypoxia. Error bars indicate ± S.E.M. * denotes $P < 0.05$ compared with the frequency difference at 300 mmHg PO_2; one way repeated measures ANOVA with Dunnett's post hoc analysis.

3.2.4 Mild mitochondrial inhibition with nitrite alters the carotid body response to hypercapnia

Hypoxia is associated with a multiplicative augmentation of the CB response to hypercapnia, showing that these two stimuli are highly interdependent (46, 185, 210). The precise site of interaction remains unknown. If hypoxic stimulus response coupling is specifically initiated through depletion of mitochondrial electron transport it would be expected that mitochondrial inhibitors, like hypoxia, are able to evoke changes in the CB sensitivity to hypercapnia.

Chemoafferent responses to hypercapnia (PCO_2 approximately 80 mmHg) of the intact CB were performed in the presence and absence of 3.3 mM NO_2^-. A mean discharge frequency in hypercapnia was taken from the final minute of exposure, after a relatively stable steady state discharge frequency had been established. An example trace of a single chemoafferent fibre response to hypercapnia in the presence and absence of 3.3 mM NO_2^- is shown in Figure 3.5A. 3.3 mM NO_2^- elevated both the basal and the frequency response to hypercapnia (Figure 3.5B). Calculations of the differences between the paired hypercapnic and basal frequencies showed that 3.3 mM NO_2^- significantly enhanced the absolute frequency rise induced by hypercapnia in all fibres tested (0.90 ± 0.20 Hz; control, 1.7 ± 0.30; nitrite) (Figure 3.5C). The calculated CO_2 sensitivity (Δ Hz / mmHg PCO_2) was significantly elevated in the presence of 3.3 mM NO_2^- (Figure 3.5D). Therefore, as in hypoxia, mild mitochondrial inhibition with NO_2^- increases the CB sensitivity to hypercapnia. This provides further evidence indicating that the impact of mitochondrial inhibition on the CB has much in common with hypoxia.

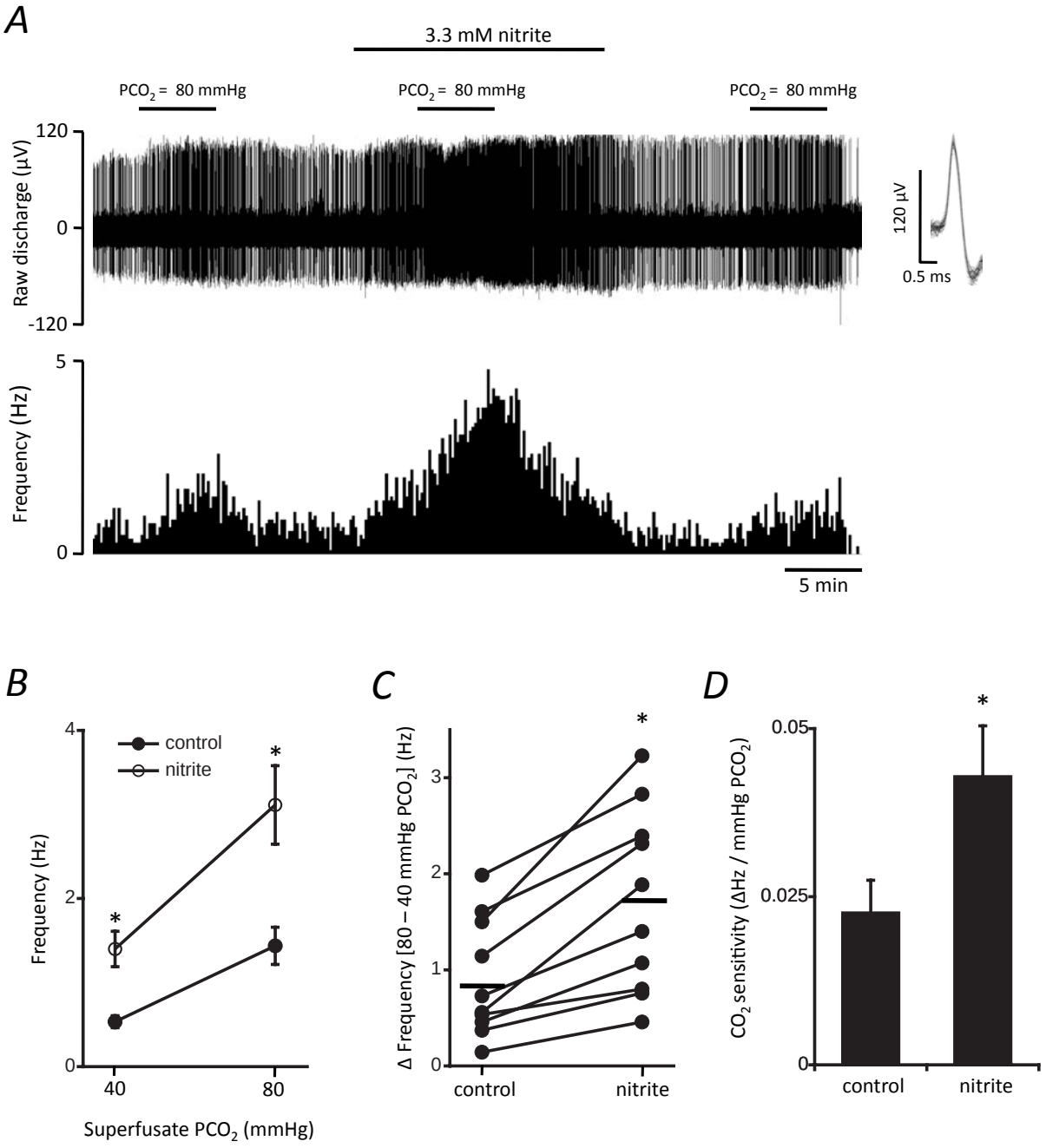


Figure 3.5 Nitrite enhances the carotid body sensitivity to hypercapnia.
The data presented was from 10 fibres from 6 CB preparations A) Characteristic example recording of the response to hypercapnia in the presence and absence of 3.3 mM nitrite (NO_2^-). Raw discharge is shown (upper) along with frequency histograms (lower) that collate single fibre action potentials in 10 s intervals. Overdrawn action potentials are shown inset to demonstrate the single fibre discrimination used to measure the frequency. B) Mean discharge frequencies recorded under normocapnic (PCO_2 = 40 mmHg) and hypercapnic (PCO_2 = 80 mmHg), in control conditions and following addition of 3.3 mM NO_2^-. Error bars indicate ± S.E.M.* denotes $P < 0.05$ compared with control group; one way repeated measures ANOVA with Bonferroni post hoc analysis. C) Discharge frequency differences (80 – 40 mmHg PCO_2) for each fibre in the presence and absence of 3.3 mM NO_2^-. D) Calculated mean CO_2 sensitivity (Δ Hz / mmHg PCO_2) in control conditions and following NO_2^- application. For D), error bars indicate + S.E.M. * denotes $P < 0.05$ compared with control group; paired t-test.

3.2.5 Carotid body chemostimulation by mild mitochondrial inhibition with nitrite is mediated through activation of AMPK

Type I cell depolarisation secondary to inhibition of mitochondrial electron transport in hypoxia has been proposed as being a consequence of either direct deactivation of background or whole cell K^+ current by a fall in $[ATP]_i$ (339) or through activation of AMPK (5, 88). Inhibition of AMPK by Compound C has been shown to depress, but not abolish, the CB chemoafferent response to acute hypoxia, indicative of hypoxia stimulus response coupling being mediated in part by AMPK (88). It is not clear whether CB excitation induced by mitochondrial inhibition is regulated by AMPK activation or through some other mechanism independent of cellular energy status, such as a change in ROS generation. Full characterisation of the downstream signalling processes in the CB secondary to impaired mitochondrial activity may be important given the link between chronic mitochondrial dysfunction and CB sLTF following CIH (2).

In the current study, 10 mM NO_2^- was applied for 5 minutes in order to achieve a measurable and reversible level of CB chemoexcitation. Following the initial response to 10 mM NO_2^-, 40 µM Compound C (dissolved in DMSO, final DMSO concentration 0.4%) was added to the superfusate for 40-50 minutes to allow for sufficient drug uptake. These concentrations of Compound C and DMSO were consistent with those that have been described previously for the intact CB preparation (88). A characteristic example trace is shown in Figure 3.6A. Inhibitory targeting of AMPK attenuated the CB basal activity and the response to 10 mM NO_2^- by approximately 42% (Figure 3.6B). Following removal of Compound C responses to moderate hypoxia remained intact indicating that the inhibition was not secondary to CB tissue damage or neurotransmitter depletion during the period of drug incubation (Figure 3.6A). For all fibres the absolute increase in

frequency induced by 10 mM NO_2^- was depressed in the presence of Compound C, with a mean reduction of approximately 39% (Figure 3.6C). These results suggested that, as with hypoxia, a component of the chemostimulation induced by mitochondrial inhibition was dependent on AMPK activation.

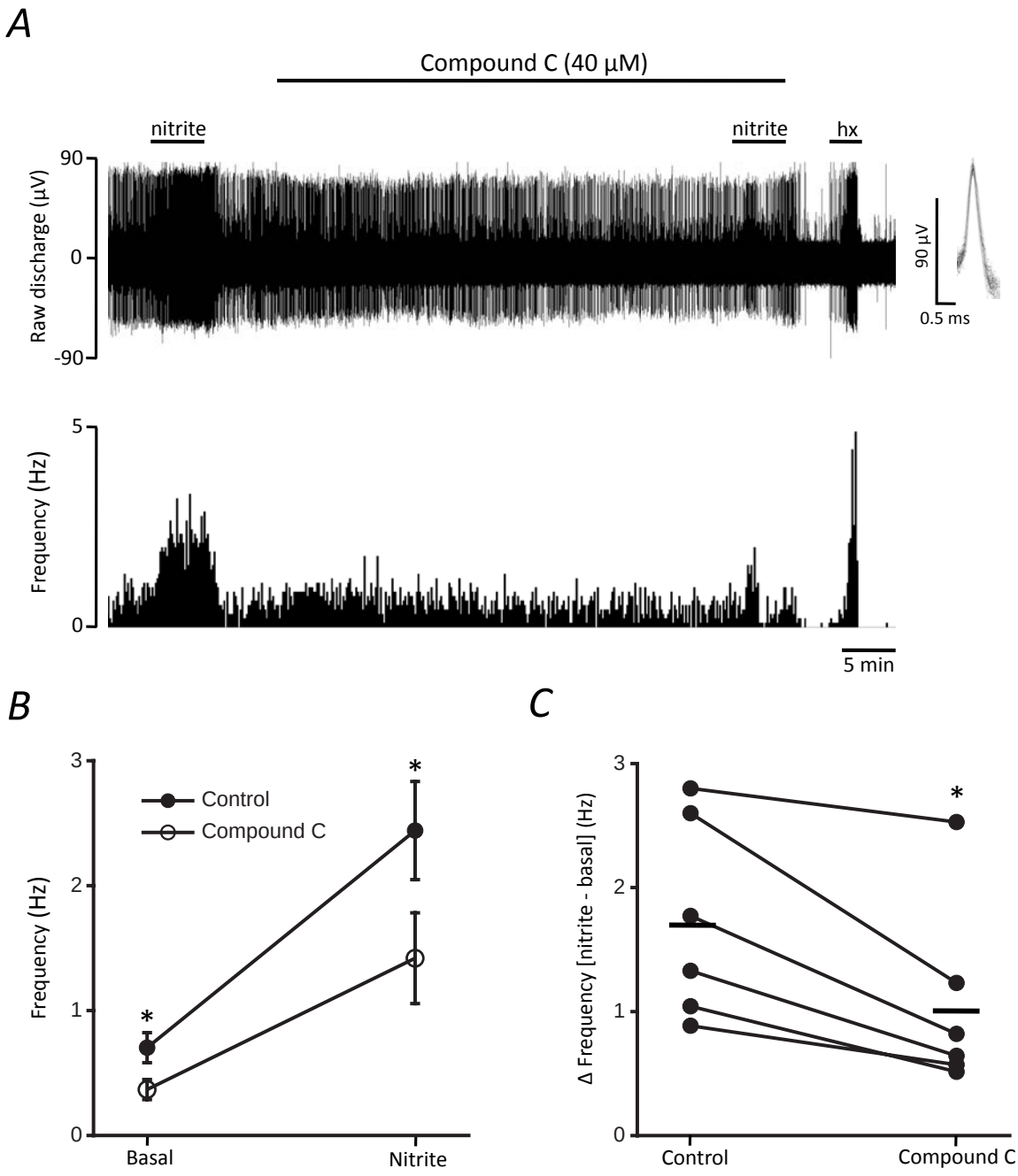


Figure 3.6 Chemostimulation evoked by mitochondrial inhibition with nitrite is dependent on AMPK activation.

The data presented was from 6 fibres from 4 CB preparations A) Characteristic example recording of the response to 10 mM nitrite (NO_2^-) in the presence and absence of the AMPK inhibitor Compound C. Raw discharge is shown (upper) along with frequency histograms (lower) that group single fibre action potentials in 10 s intervals. Overdrawn action potentials are shown inset to demonstrate the single fibre discrimination used to measure frequency. B) Mean discharge frequencies recorded in basal conditions and in response to 10 mM NO_2^-, in the presence and absence of Compound C. Error bars indicate ± S.E.M.* denotes $P < 0.05$ compared with control group; one way repeated measures ANOVA with Bonferroni post hoc analysis. C) Discharge frequency differences (NO_2^- - basal) for each fibre in the presence and absence of Compound C. * denotes $P < 0.05$ Compound C compared with control group; paired t-test.

3.3 Chapter synopsis and discussion

3.3.1 Summary of key findings

The main findings of the current chapter are described as follows;

- Mild mitochondrial inhibition using exogenous NO_2^- changed the set point for hypoxia stimulus response coupling in the CB and therefore for the first time provides direct evidence of a significant interaction between these two stimuli. In this way these data strongly support the hypothesis that CB hypoxia sensing is influenced by a reduction in mitochondrial energy metabolism.

- The PO_2 dependent action of NO_2^- on type I cell stimulation indicates that NO_2^- induced mitochondrial inhibition and CB stimulation is a consequence of NO generation. This suggests that in the CB type I cell, NO has the potential to directly modulate mitochondrial function and type I cell excitability.

- Mitochondrial inhibition with NO_2^-, as with hypoxia, sensitised the CB to hypercapnic stimulation.

- Chemostimulation evoked by mitochondrial inhibition with NO_2^-, as with hypoxia, is partly blocked by Compound C, an inhibitor of AMPK.

3.3.2 Mild inhibition of mitochondrial function changes the set point for hypoxia stimulus response coupling in the carotid body

A number of studies have identified similarities in transduction processes between hypoxia and mitochondrial inhibition induced chemostimulation, specifically those being deactivation of background K^+ (TASK-like and TREK-1) currents (54), Ca^{2+} influx through L-type Ca^{2+} channels (54), neurosecretion (52) and stimulation of the CSN afferents (50-52, 337). Furthermore, CB type I cell mitochondrial function seems to be more sensitive to a fall in PO_2 than other cell types showing a reduction in mitochondrial electron transport and mitochondrial depolarisation at PO_2 levels as high as 40 mmHg (4, 56).

The main focus of the current chapter was to identify the presence of any response interaction between mild mitochondrial inhibition and hypoxia in the mammalian CB. Evaluation of the impact of mild mitochondrial inhibition on CB hypoxic sensitivity over a range of different PO_2s has not been examined previously in this tissue. For mitochondrial inhibition to be further implicated in CB hypoxia stimulus response coupling, when the two stimuli were applied concurrently, they would ideally produce multiplicative responses. Here, by using NO_2^- as a novel and rapidly reversible inhibitor of mitochondrial metabolism, it was demonstrated that under conditions of mild NO_2^- induced basal CB stimulation, the subsequent hypoxic sensitivity of the CB was significantly augmented. Analysis of the hypoxic response curves showed that mild mitochondrial inhibition with NO_2^- changed the ‘set point’ of hypoxic response initiation, suggesting that the level of mitochondrial function is central in setting the threshold for CB hypoxic stimulation. Inspection of the absolute increase in chemoafferent activity evoked by NO_2^- over a full range of PO_2 levels showed that this elevation was further

increased in hypoxia. This was indicative of a strong multiplicative interaction between mitochondrial inhibition and hypoxic stimuli and ruled out the notion that these stimuli act independently. These data are consistent with the hypothesis that CB hypoxic sensing is critically dependent on a run-down in mitochondrial function.

Recently, support for H_2S in mediating the CB response to hypoxia has received much attention. This is based on experiments that have demonstrated an elevation in both CSN discharge frequency and DA secretion in response to high doses of exogenous H_2S donors (131, 132). A role for H_2S is further strengthened by observations showing that functional CB hypoxic responses are attenuated, but not abolished in cystathione γ lyase (an enzyme capable of generating H_2S) deficient mice (132). It has been proposed that the direct target of H_2S in type I cells is the BK_{Ca} channel and H_2S concentrations of 40 μM and above have been shown to inhibit K^+ conductance of this channel (133). However, it has also been demonstrated that exogenous H_2S concentrations similar to those that stimulate the CB and deactivate the BK_{Ca} channel also produce intense inhibition of mitochondrial function in type I cells (130). In view of the findings from this chapter, whilst a role for H_2S in CB hypoxic sensing cannot be ruled out, it is proposed that its actions are most probably a consequence of a reduction in mitochondrial energy metabolism and activation of signalling pathways that are dependent on a depleted/altered cellular energy status.

3.3.3 The use of exogenous nitrite as a novel type I cell mitochondrial inhibitor

The use of exogenous NO_2^- in the current study was not only because it has recently been shown to reversibly impair mitochondrial function in other tissues, but also because this mechanism is thought to be regulated specifically through generation of

NO (172, 176, 179). Despite NO being known to reversibly inhibit mitochondrial electron transport through binding to cytochrome c oxidase in other cells (177), the potential modification of mitochondrial function by NO in the CB type I cells has not been examined before. An effect of NO on the mitochondria may have been masked in previous studies on the CB (using other NO donors or NOS blockers) because of its known inhibitory actions at other sites within the type I cell (148, 149).

In the present experiments very high supraphysiological concentrations of NO_2^- evoked dose dependent elevations in chemoafferent discharge frequency; an effect that was coupled to dose dependent mitochondrial inhibition in isolated type I cells as evidenced by rises in NADH autofluorescence. Therefore, as in other cell types, NO_2^- was shown to depress mitochondrial electron transport.

It is proposed that the action of NO_2^- on the mitochondria was a consequence of NO production. The formation of NO from NO_2^- was not measured and NO scavengers were not used because of their limited cell permeability (340). However, it was observed that the impact of NO_2^- on type I cell $[Ca^{2+}]_i$ responses was critically dependent on O_2, with NO_2^- only evoking rises in $[Ca^{2+}]_i$ at a dissolved O_2 concentration of 2% and not 20%. Given that the reduction of NO_2^- to form NO and the NO binding to cytochrome c oxidase are both favoured in hypoxia (172, 179), these current results are consistent with the excitatory effect of NO_2^- being a consequence on the formation and action of NO. Future studies should be performed to measure NO production directly upon addition of NO_2^- within each cellular compartment of the type I cell, to confirm that these excitatory actions of NO_2^- are NO dependent.

If the impact of NO_2^- on CB function was through production of NO, it may be plausible to question why the well characterised inhibitory actions of NO on CB activity were not observed here. It is hypothesised that NO_2^- itself was reduced at the site of the mitochondria and only generated local rises in NO that were limited to the mitochondrial compartments. Mitochondrial reduction of NO_2^- into NO has been shown to only occur when supraphysiological (mM) concentrations of NO_2^- are applied exogenously (172-174). Accordingly, the inhibition of mitochondrial function and elevation in chemoafferent discharge were only detected in these experiments when at least 3.3 mM NO_2^- was applied to the superfusate, indicative of selective mitochondrial dependent NO_2^- reduction and NO generation. Cytosolic NO_2^- reductase activity in cardiac or smooth muscle cells has been shown to take place at physiological µM concentrations of NO_2^- (154, 175, 180, 341). It is speculated that the number of type I cell cytosolic NO_2^- reductases was not sufficient to generate enough cytosolic NO to inhibit L-type Ca^{2+} current or activate BK_{Ca} channels, and for this reason no inhibitory effects of NO were observed in the presence of NO_2^-.

3.3.4 A role for endogenous nitric oxide in modulating type I cell mitochondrial function

The evidence described in the current chapter indicates that only supraphysiological concentrations of exogenous NO_2^- are able to reduce type I cell mitochondrial activity. It is unlikely that enough NO can be generated from endogenous concentrations (µM) of NO_2^- to be functionally active in the CB. However, the results also imply that if enough NO is produced locally at the mitochondria then it has the potential to reduce mitochondrial energy metabolism and sensitise the CB to hypoxia. Since measurable amounts of NO have been identified in mitochondrial membranes of type I cells following

hypoxia, it is possible that NO may be available endogenously to alter mitochondrial function (83).

The source of this endogenous NO is most likely to be from NOS-3 given that this is the only NOS isoform to be detected in the type I cell (83). Accordingly, Kline and colleagues identified that NOS-3 deficient mice had a significantly diminished ventilatory response to hypoxia; an observation that was coupled with a downgraded CB function (144). It was hypothesised that chronic vasoconstriction of the arterial supply to the CB would have subjected the organ to chronic hypoxia from birth. Since chronic neonatal hypoxia is known to be associated with an impairment of the CB tissue to develop hypoxic chemosensitivity (45, 145), the authors concluded that this was the sole reason for the reduction in CB function. Whilst this is a plausible explanation, in view of the current findings, it is proposed that maybe a component of the inhibition of CB hypoxic sensitivity was a consequence of the absence of NOS-3 derived NO and its action on type I cell mitochondria. In conclusion, it is suggested that NO may have dual effects on type I cell function; an inhibitory action exerted through modulation of ion channels and a novel stimulatory action achieved through regulation of mitochondrial electron transport. In this respect it may be similar to the previously proposed dual actions of CO (109, 110, 116, 120).

3.3.5 Mitochondrial inhibition with nitrite sensitised the CB to hypercapnic stimulation

An interaction between hypoxic and hypercapnic stimuli leading to the generation of multiplicative responses has been observed previously in the CB (46, 210). Data from the current study suggests that mild mitochondrial inhibition with NO_2^- was capable of

significantly augmenting the absolute chemoafferent frequency response of the CB to subsequent hypercapnic stimulation. Thus, for the first time in the CB, a multiplicative interaction between mitochondrial inhibition and hypercapnia was demonstrated. This provided further evidence suggesting that mitochondrial inhibition and hypoxic stimuli have very similar actions on CB chemoreceptor function.

The exact site of interaction between mitochondrial inhibition and hypercapnic stimuli was not investigated in this study. These stimuli share a number of key transduction processes including attenuation of background TASK-like conductance, activation of L-type Ca^{2+} channels, neurosecretion and stimulation of the CSN afferents (18). Potential interaction could be a consequence of up-regulation of any or all of these processes. However, it is also known that NO_2^- reduction is favoured in acidic conditions. Therefore, in the current investigation, it is possible that the generation of NO was increased during the acidic hypercapnic conditions, causing further impairment of mitochondrial activity and chemostimulation. Nevertheless, the data still supports a role for the mitochondria in setting not only the CB threshold for the response to hypoxia but also to hypercapnia. Whether it may set the threshold to other putative stimuli of the CB is not known.

3.3.6 Carotid body chemoafferent stimulation produced by mitochondrial inhibition with nitrite is regulated by activation of AMPK

The CB chemoafferent response to hypoxia is recognised as being attenuated by the AMPK inhibitor Compound C (88). This suggests that a degree of hypoxia induced metabolic stress may be necessary for activating the downstream signalling pathways associated with type I cell stimulation. In this study, Compound C also reduced the chemoafferent response to NO_2^-, in every fibre tested. The magnitude of this response

depression was consistent with that described previously for hypoxia (88). This indicates that, as in hypoxia, the type I cell response to mitochondrial inhibition is dependent on a change in cellular energy status and activation of AMPK.

CB hypersensitivity and a baseline elevation in chemoafferent outflow is associated with mitochondrial dysfunction in animals pre-conditioned with CIH (313). Based on the novel associations between mitochondrial impairment, AMPK activation and chemoafferent stimulation identified in this chapter, it is proposed that a proportion of the CB hypersensitivity following CIH is likely to be mediated through an increase in AMPK activity in the type I cell. Future investigations could examine this directly by characterising the phosphorylation status of AMPK and its downstream target acetyl CoA carboxylase following periods of CIH. Furthermore, pharmacological inhibition of AMPK using Compound C could be used to more clearly define a functional role for AMPK in evoking CB sLTF following CIH.

The results presented in this chapter also demonstrate that a significant component of the chemoafferent response to mitochondrial inhibition with NO_2^- was still preserved in the presence of Compound C. This may have been because of incomplete deactivation of AMPK as a consequence of the restricted uptake or activity of Compound C. 40 µM Compound C was used as a pharmacological inhibitor of AMPK as has been described previously in both whole CB tissue and single cell hepatocyte preparations (88, 342). In the single cell preparations 40 µM Compound C has been shown to deactivate AMPK activity by up to 75% following exposure to AICAR or metformin (342). In whole CB preparations the response to hypoxia, following 40 µM Compound C incubation, was reduced (by approximately 50%) but not abolished (88). Therefore, the inhibitory impact

of Compound C on the CB chemoafferent stimulation by mitochondrial inhibition observed in this study was similar to that previously seen in hypoxia.

Alternatively, a significant element of the stimulation evoked by mitochondrial inhibition may have been dependent on a reduction in $[ATP]_i$ and direct inhibition of the ATP sensitive TASK-like channel K^+ current (343). Another possibility is that the impairment of mitochondrial function by NO_2^- may have activated signalling pathways dependent on ROS formation. Preliminary experiments performed in our laboratory have demonstrated that the chemoafferent frequency increase induced by NO_2^- was preserved in the presence of the superoxide scavenger Tempol (1 mM), indicative of the response to NO_2^- not being mediated through increased ROS generation (Supplementary figure 2).

A dose of 40 μM Compound C was used to inhibit AMPK. Compound C has been shown to deactivate AMPK by altering the configuration of the α subunit phosphorylation activation loop (344). Compound C was dissolved in DMSO (final DMSO concentration 0.4%) and the CB incubation time in Compound C was between 40–50 minutes as described previously (88). To investigate if the inhibitory effect observed following incubation in Compound C and DMSO was a consequence of tissue damage or neurotransmitter depletion, a mild hypoxic stimulus was applied following removal of the drug and solvent from the superfusate. In all experiments the chemoafferent responses to hypoxia were greater than that observed in response to the original NO_2^- stimulation (See example in Figure 3.6A). It is suggested that the CB tissue was still viable and retained normal chemoreceptor function and that the suppression of the response to NO_2^- was a consequence of the pharmacological inhibition of AMPK activity.

Because of the potential limitations of Compound C to completely deactivate AMPK, some of subsequent studies described in this thesis were performed on CBs isolated from animals with partial or complete knock down in Lkb1 (the upstream kinase required for AMPK activation). This was done in order to more clearly define a role for the Lkb1-AMPK signalling pathway in CB hypoxia stimulus response coupling.

3.3.7 Conclusions

Chemoafferent responses evoked by the novel type I cell mitochondrial inhibitor NO_2^- are comparable with those induced by hypoxia. Mild mitochondrial inhibition with NO_2^- significantly enhanced the CB sensitivity to hypoxia. This previously uncharacterised finding suggests that the sensing of hypoxia in the CB is closely associated with a hypoxia evoked impairment of mitochondrial electron transport. This has the potential to induce cellular metabolic stress leading to the direct activation of AMPK. However, the precise mechanism that links a reduction in mitochondrial energy metabolism with type I cell stimulation needs to be evaluated further.

4. Does the Lkb1-AMPK signalling pathway provide the key to carotid body activation by hypoxia?

4.1 Chapter introduction and overview

Studies performed in the previous chapter supported the hypothesis that CB hypoxia stimulus response coupling is a consequence of a run-down in mitochondrial energy metabolism. A definitive signalling process that links the reduction in mitochondrial function with type I cell depolarisation and stimulation remains unidentified. With this in mind, the primary focus of the current chapter was to investigate whether activation of the Lkb1-AMPK pathway is a necessary intermediate step in mediating this hypoxia chemotransduction process.

It is hypothesised that depletion of mitochondrial electron transport in the type I cell has the potential to directly evoke local or whole cell changes in either $[ATP]_i$ or the AMP:ATP ratio subsequent to adenylate kinase activation. A decrease in the $[ATP]_i$ in hypoxia has the potential to depolarise the type I cell by directly reducing the ATP sensitive background TASK-like channel current (343). It has also been proposed that type I cell hypoxia stimulus response coupling may be conferred through changes in the AMP:ATP ratio via AMPK, a ubiquitously expressed protein kinase and sensor of cellular energy status (6, 88).

It is now recognised that the overall activity of AMPK is augmented by approximately 1000 fold as a consequence of AMP binding at the two available CBS domains on the

regulatory γ subunit (93, 96). This AMP mediated increase in AMPK activation is achieved through a combination of direct allosteric activation and the promotion of Thr 172 residue phosphorylation in the kinase activation loop on the catalytic α subunit (90, 96). The latter is thought to be dependent on the up-regulation of upstream kinase activity (95) and a reduction in PP2C phosphatase activity (97). ATP is also capable of binding at the two available CBS domains on the γ subunit but, in contrast to AMP, this binding reduces the extent of AMPK phosphorylation and limits activation (93, 97, 100). The overall level of AMPK activation seems to be dependent on the cellular AMP:ATP ratio (6). A number of pharmacological and physiological stimuli, associated with increasing the AMP:ATP ratio, activate AMPK. These include mitochondrial poisons (101), glucose deprivation (103), skeletal muscle contraction (102), regional ischaemia (104) and hypoxia (5).

In mammalian cells Lkb1 has been identified as the predominant upstream kinase that phosphorylates and activates AMPK (7). In certain cell types the calmodulin dependent kinase kinase beta (CaMKKβ), has been expressed as an alternative kinase capable of phosphorylating AMPK in response to elevated $[Ca^{2+}]_i$ (345). Lkb1 forms a heterotrimeric complex with STRAD and MO25 and direct interactions with these two regulatory proteins augment the overall Lkb1 activity, thereby promoting the phosphorylation of Thr 172 on the α subunit of AMPK (98, 99). It has now been established that sufficient levels of Lkb1 are required to activate AMPK during muscle contraction, ischaemia and hypoxia (106, 346).

In the CB, the α1 subunit of AMPK is positively expressed in the type I cells and is co-localised with the BK_{Ca} channel at the plasma membrane (5, 88). Pharmacological

activation of AMPK with AICAR elevates the type I cell $[Ca^{2+}]_i$ and increases the CSN discharge frequency (5). Thus, activation of AMPK mimics, to a certain degree, the actions of hypoxia. A number of specific AMPK phosphorylation targets in the type I cell have been identified including the TASK, TREK-1 and BK_{Ca} channels, all of which are associated with CB hypoxia chemotransduction (29, 54, 88). Inhibition of AMPK with Compound C also virtually abolishes the hypoxia induced elevation in type I cell $[Ca^{2+}]_i$ and reduces the increase in CSN chemoafferent discharge frequency by approximately 50% (88). Finally, respiratory measurements indicate that mice with global deletion of the gene encoding the α2 subunit of AMPK have an impaired hypoxic ventilatory response (108).

The work performed by Evans and colleagues regarding the role for AMPK in CB hypoxic chemotransduction has been viewed with a high degree of caution. This is primarily because the physiological actions of both AICAR and Compound C may be exerted through kinases independent of AMPK activation (347). The dose of 1 mM AICAR, used by Wyatt et al. (88) potentially may also have enhanced the chemoafferent activity by elevating synaptic adenosine concentrations subsequent to the saturation of the bidirectional ENT adenosine transporter, although this is yet to be confirmed. In addition, even though the ventilatory response to hypoxia was reduced in the α2 AMPK KO mice, a significant element was still preserved. Finally, the inability of Compound C to completely abolish the increase in CSN frequency evoked by hypoxia (88) suggests that AMPK activation may only be a single component of the complete hypoxia transduction process and/or Compound C does not completely deactivate AMPK.

The experiments presented in the current chapter were performed on CBs isolated from animals with partial or complete knock down in the Lkb1, the essential upstream kinase and activator of AMPK (7). This was done to more clearly establish or reject a role for the Lkb1-AMPK signalling pathway in CB hypoxia stimulus response coupling.

Potential changes in the hypercapnic sensitivity of CBs deficient in Lkb1 were also investigated. Any alteration in hypoxic sensitivity may be expected to impact on the response to hypercapnia given the strong degree of synergy known to exist between these two stimuli (46, 210).

The aims of the investigations in this chapter are summarised below:

1. To investigate if Lkb1-AMPK signalling is necessary for the generation of basal CB chemoafferent activity.

2. To establish if progressive depletion of Lkb1 correlates with a progressive depression in the CB response to hypoxia.

3. To examine if Lkb1-AMPK signalling is essential for CB stimulation by hypercapnia.

4.2 Results

4.2.1 Mouse model development and generation of carotid body type I cells deficient in Lkb1

All breeding and genetic development of mice was performed at the University of Edinburgh and not by the author of this thesis. Transgenic C57BL/6J mice with the loxP excision sequence flanking the stk11 gene encoding Lkb1 on each chromosome 10 were termed $Lkb1^{fl/fl}$ or Lkb1 *flox*. Transgenic C57BL/6J mice homozygous for the gene that encoded the Cre recombinase protein under the tyrosine hydroxylase promoter (Th-IRES-Cre) were supplied by the European Mutant Mouse Archive (EMMA) and were termed $Cre^{+/+}$ or TH-Cre.

First generation crossing of the $Lkb1^{fl/fl}$ with the $Cre^{+/+}$ mice generated a population of mice heterozygous for both the loxP excision sequence (flanking the stk11 gene) and the gene encoding Cre recombinase enzyme and these mice were termed $Lkb1^{fl/-}Cre^{+/-}$. Crossing of these second generation mice generated multiple genetically different mice populations, two of which were of interest. The first was heterozygous for the loxP excision sequence flanking the stk11 gene on chromosome 10 and homozygous for the gene encoding Cre recombinase enzyme. These mice were termed $Lkb1^{fl/-}Cre^{+/+}$ or Lkb1 het KO and were estimated to have a 50% reduction in Lkb1 protein in cells expressing TH. The second group of interest was homozygous for the loxP excision sequence and homozygous for the gene encoding Cre recombinase enzyme. These mice were termed $Lkb1^{fl/fl}Cre^{+/+}$ or Lkb1 hom KO and were predicted to have a complete knock down in Lkb1 protein expression in TH positive cells. A simplified version of breeding patterns used to generate these mice is shown in Figure 4.1.

Since global knock down of Lkb1 is embryonic lethal (346), Cre-Lox recombination technology was used to ensure that only cells expressing TH were partially or totally deficient in Lkb1. In the Lkb1 hom KO ($Lkb1^{fl/fl}Cre^{+/+}$) mice for example, because the Cre recombinase gene was under the TH promoter (THRES-Cre), whenever TH was expressed so was Cre recombinase enzyme. Cre recombinase subsequently recognised the loxP excision sequence and deleted the flanked gene (in this case stk11), completely preventing the expression of Lkb1 in these TH positive cells.

mRNA analysis from single type I cells revealed that stk11 gene expression was completely absent in the Lkb1 hom KO ($Lkb1^{fl/fl}Cre^{+/+}$) mice but was positively detected in WT C57BL/6J mice strains (data not shown). In the TH positive adrenal chromaffin cells, Lkb1 protein expression in Lkb1 hom KO ($Lkb1^{fl/fl}Cre^{+/+}$) was almost completely abolished compared with TH-Cre ($Cre^{+/+}$) controls (data not shown). All single cell RT-PCR and protein quantification assays were performed by colleagues at the University of Edinburgh and not by the author of this thesis. These data provide confirmatory evidence of Lkb1 hom KO mice being deficient in Lkb1 in TH positive cells.

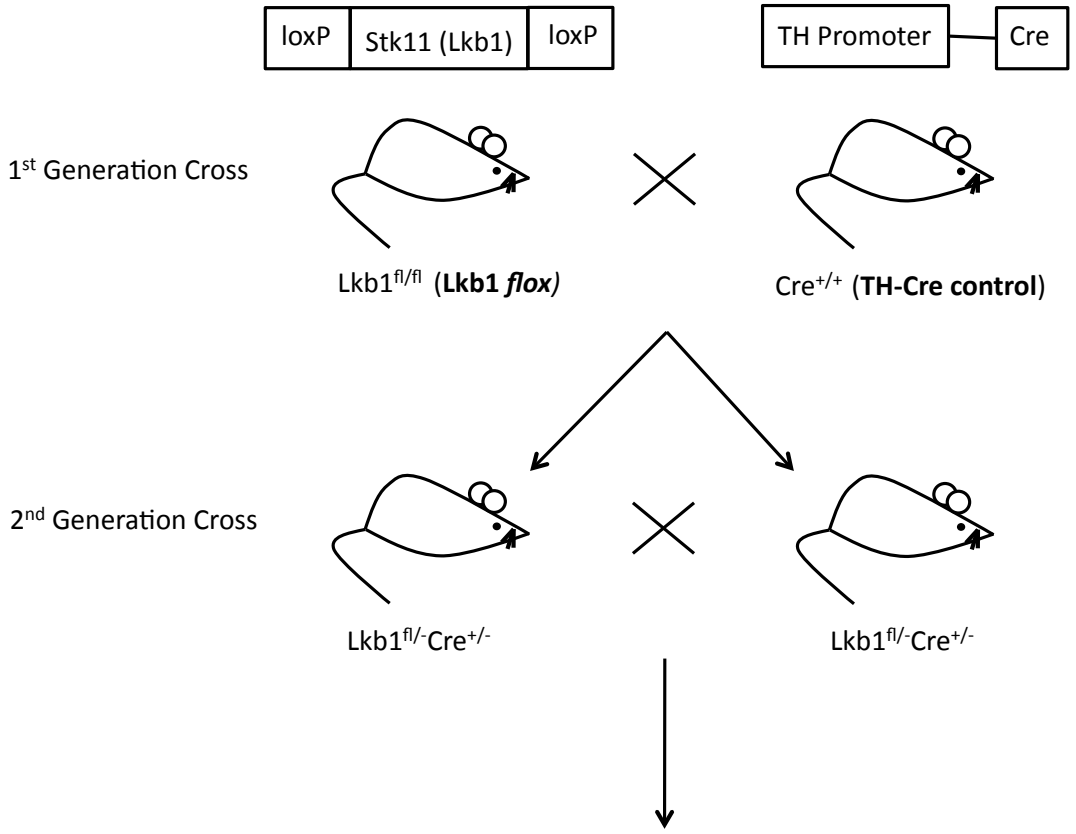


Figure 4.1 A simplified schematic of the breeding patterns used to generate the first generation of mice partially or totally deficient in Lkb1 in tyrosine hydroxylase (TH) expressing cells.

4.2.2 Deletion of Lkb1 in carotid body type I cells virtually abolishes the chemoafferent response to hypoxia

In vitro recordings of the chemoafferent activity were made from CBs isolated from 4 different mouse groups; TH-Cre ($Cre^{+/+}$), Lkb1 *flox* ($Lkb1^{fl/fl}$), Lkb1 het KO ($Lkb1^{fl/-}Cre^{+/+}$) and Lkb1 hom KO ($Lkb1^{fl/fl}Cre^{+/+}$). These groups were chosen as they had a proposed progressive depletion of type I cell Lkb1 mRNA and protein expression. Previous studies have reported that Lkb1 *flox* mice had a 5-10 fold reduced expression of Lkb1 compared with wild type littermates (106, 346). Therefore, in this study, the TH-Cre group was taken as the only control.

Initial experiments were performed to detect any potential alterations in basal chemoafferent activity and hypoxic sensitivity in CBs deficient in Lkb1. Basal activity was monitored at a superfusate PO_2 of approximately 200 mmHg and a PCO_2 of approximately 40 mmHg. To induce responses to hypoxia, the superfusate PO_2 was slowly reduced to a minimum of 40 mmHg or was reversed prior to this when the chemoafferent response had stabilised or had begun to diminish. Characteristic raw trace examples for all mouse groups are shown in Figure 4.2 and demonstrate that CB single fibre chemoafferent responses to hypoxia were depressed according to the extent of type I cell Lkb1 depletion.

Analysis of grouped data revealed that the basal single fibre chemoafferent discharge frequency was not significantly different between the TH-Cre and the Lkb1 *flox* groups measuring 0.69 ± 0.21 Hz and 0.72 ± 0.14 Hz respectively (Figure 4.3A and B). The basal activity was significantly depressed in the Lkb1 het KO and Lkb1 hom KO groups, and by the same magnitude, compared with the TH-Cre and the Lkb1 *flox* groups,

measured as 0.20 ± 0.05 Hz and 0.20 ± 0.04 Hz respectively (Figure 4.3A and B). Taken together, these data indicate that a degree of Lkb1 expression was highly significant, but not completely essential, for the generation of basal CB chemoafferent activity.

Analysis of the responses to hypoxia showed that the absolute peak single fibre discharge frequency recorded in severe hypoxia was significantly attenuated in the Lkb1 *flox* group (7.26 ± 1.00 Hz) compared with the TH-Cre control (13.00 ± 1.51 Hz) (Figure 4.3C). This was further significantly suppressed in CBs from Lkb1 het KO (4.17 ± 0.34 Hz) and Lkb1 hom KO (2.84 ± 0.78 Hz) mice (Figure 4.3C). Although the magnitude of the mean peak hypoxic frequency tended to be smaller in the Lkb1 hom KO group compared with the Lkb1 het KO group, no statistical difference was observed between these two groups (Figure 4.2 and 4.3C).

Mean hypoxic response curves were constructed by plotting the calculated discharge frequency against a range of defined PO_2 levels. Statistical analysis revealed that the shape of chemoafferent hypoxic response curves obtained from CBs partially or completely deficient in Lkb1 were significantly different compared with the TH-Cre controls ($P < 0.05$; Two way repeated measures ANOVA with Bonferroni post hoc comparisons test) (Figure 4.3A). Hypoxic response curves plotted for Lkb1 het KO and Lkb1 hom KO groups were also significantly different compared with the Lkb1 *flox* group ($P < 0.05$; Two way repeated measures ANOVA with Bonferroni post hoc comparisons test) (Figure 4.3A). Although the mean single fibre discharge frequency recorded from the Lkb1 hom KO group was reduced at every PO_2 value compared with the Lkb1 het

KO group, the overall response to hypoxia showed no statistical difference (Figure 4.3A).

Analysis of specific parameters of these hypoxic response curves identified that the rate of increase in discharge frequency with decreasing PO_2 was depressed in Lkb1 hom KO mice CBs as evidenced by the mean exponential rate constant being significantly smaller than that calculated from the TH-Cre group (0.024 ± 0.007 v 0.041 ± 0.006) (Figure 4.4A). The PO_2 recorded when the peak discharge frequency had been attained was significantly lower in Lkb1 hom KO group compared with TH-Cre controls (60 ± 3 mmHg v 71 ± 2 mmHg), indicative of a marked ‘left shift’ in the hypoxic response curve (Figure 4.4B). Both of these factors may account for the relative ‘flattening’ and ‘left shift’ of Lkb1 hom KO hypoxic response curve observed in Figure 4.4A. These observations showed that Lkb1 depletion severely diminished the CB peak frequency response to hypoxia, changed the PO_2 set point required for CB hypoxic response initiation and significantly attenuated the CB hypoxic sensitivity. These findings strongly support the hypothesis that Lkb1-AMPK signalling is required for CB activation by hypoxia.

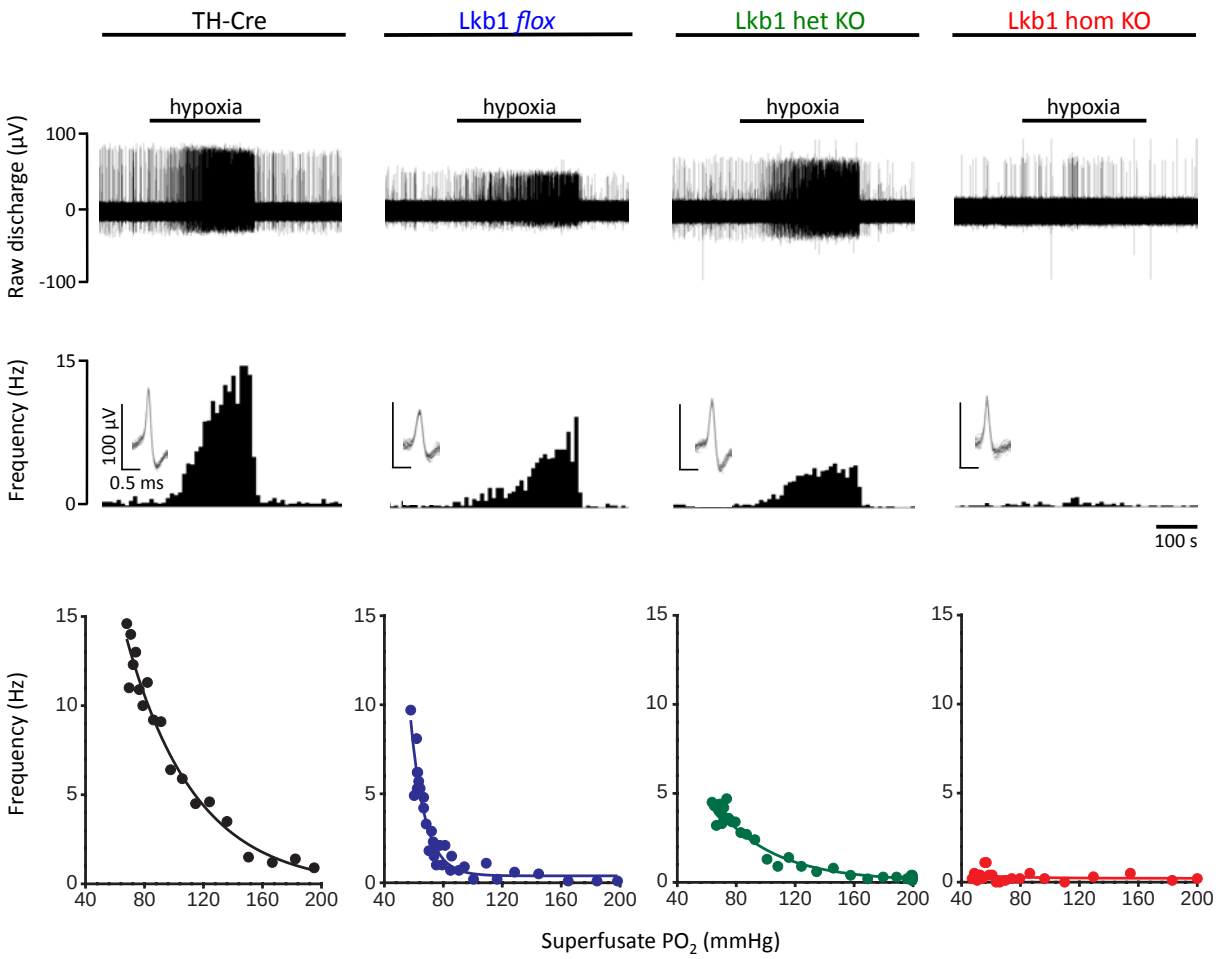


Figure 4.2 Sequential deletion of Lkb1 progressively attenuates the carotid body response to graded hypoxia.
Upper: Example raw traces of single/few fibre *in vitro* extracellular chemoafferent recordings during exposure to graded hypoxia for each transgenic mouse group: TH-Cre (control), Lkb1 *flox*, Lkb1 het KO and Lkb1 hom KO. Frequency time histograms (middle) and frequency PO_2 response curves (lower) for each example trace are shown, with a progressive decrease in hypoxia sensitivity observed with incremental deletion of Lkb1. Single fibre discriminations is shown in middle panel insets.

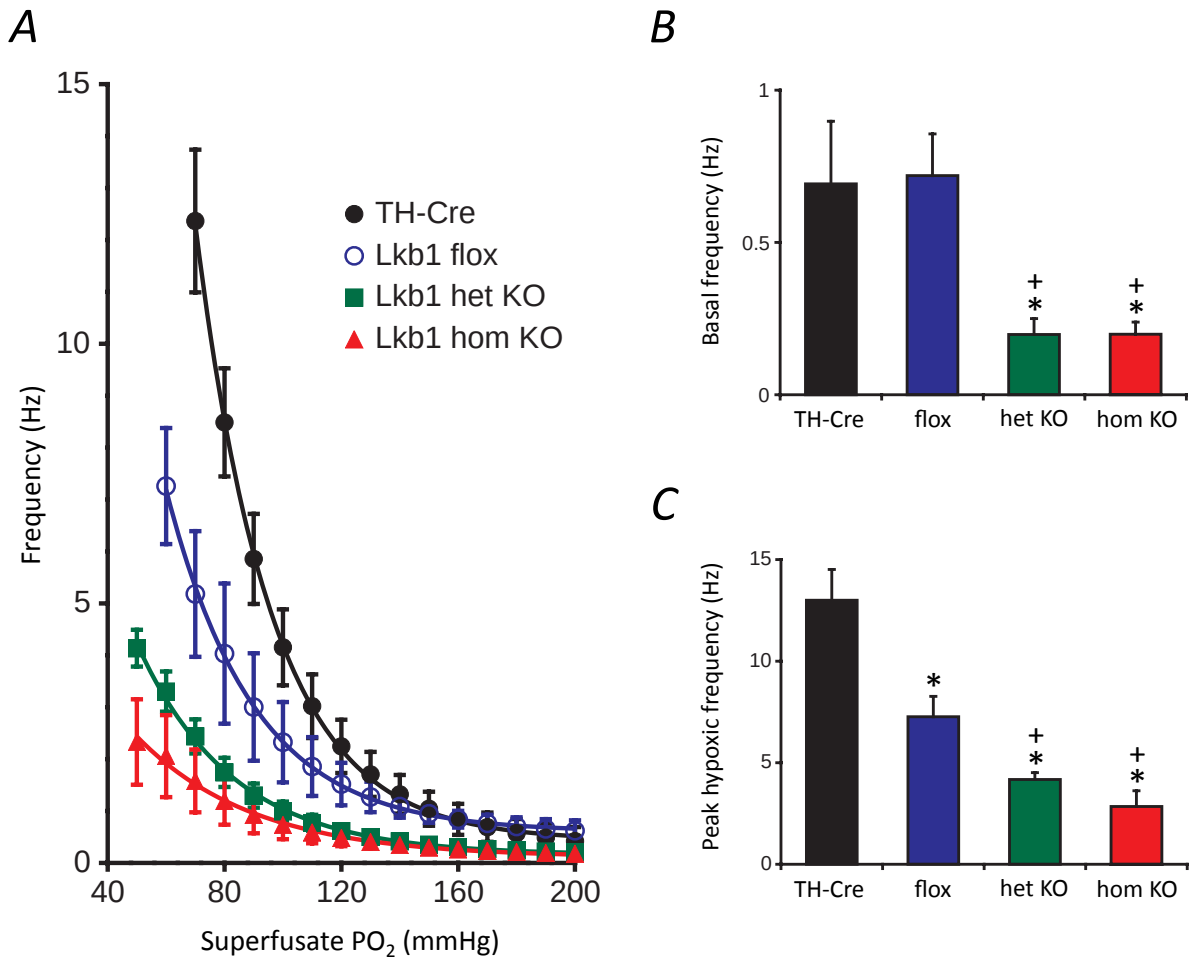


Figure 4.3 Lkb1 deletion impairs basal chemoafferent activity and diminishes the hypoxic sensitivity of the *in vitro* mouse carotid body.
A) Mean hypoxic response curves for each of the 4 transgenic mouse groups as follows: TH-Cre control; n=8 fibres from 6 mice, Lkb1 *flox*; n=8 fibres from 7 mice, Lkb1 het KO; n=12 fibres from 8 mice and Lkb1 hom KO; n=7 fibres from 5 mice. Single fibre discharge frequency is plotted against PO_2 between basal discharge to a maximum mean frequency plateau or failure. Mean response curves *for* Lkb1 *flox*, Lkb1 het KO and Lkb1 hom KO were significantly different to the TH-Cre (control) curve ($P < 0.05$) and Lkb1 het KO and Lkb1 hom KO hypoxic response curves were significantly different to the Lkb1 *flox* response curve ($P < 0.05$); two way repeated measures ANOVA with Bonferroni post hoc comparisons test.
B) Mean basal single fibre discharge frequency observed for each of the 4 transgenic mouse groups. Error bars indicate mean + S.E.M. * denotes $P < 0.05$ vs TH-Cre and + denotes $P < 0.05$ vs Lkb1 *flox*; one way factorial ANOVA with Bonferroni post hoc comparisons test.
C) As in B) but for the single fibre peak discharge frequency attained in hypoxia. Error bars indicate mean + S.E.M. * denotes $P < 0.05$ vs TH-Cre and + denotes $P < 0.05$ vs Lkb1 *flox*; one way factorial ANOVA with Bonferroni post hoc comparisons test.

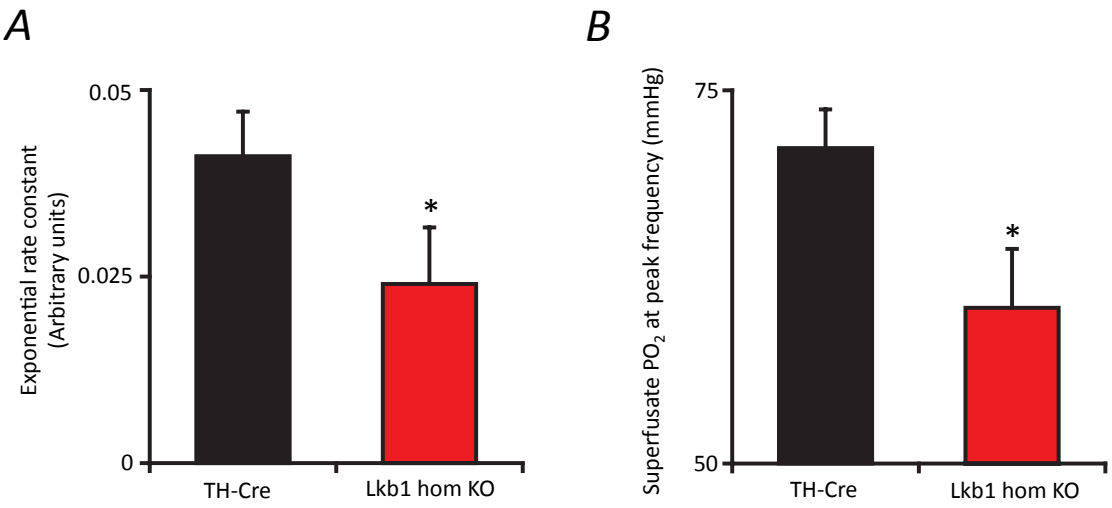


Figure 4.4 Lkb1 deletion depresses the carotid body hypoxic response curve exponential rate constant and lowers the PO_2 at which the maximum discharge frequency is achieved.
A) Mean exponential rate constants calculated from hypoxic response curves from TH-Cre (control; n=8 fibres from 6 mice) and Lkb1 hom KO (Lkb1 hom KO; n=7 fibres from 5 mice) groups. Error bars indicate mean + S.E.M. * denotes $P < 0.05$ vs TH-Cre; unpaired t-test. B) As in A) but for the superfusate PO_2 measured at the point when the peak discharge frequency had been attained.

4.2.3 Deletion of Lkb1 in carotid body type I cells attenuates the chemoafferent response to hypercapnia

Significant multiplicative CSN response interactions measured during simultaneous hypoxic and hypercapnic stimulation have been described previously (46). In Section 4.2.2, it was demonstrated that partial or complete deletion of type I cell Lkb1 markedly diminished the chemoafferent response to hypoxia. It was next examined whether the CBs isolated from Lkb1 hom KO mice, that had a significant down-regulation in hypoxic sensitivity, had a suppressed chemoafferent response to hypercapnia, as would be expected if these stimuli were interdependent.

Chemoafferent responses to hypercapnia (PCO_2 approximately 80 mmHg, PO_2 approximately 200 mmHg) were performed on CBs isolated from TH-Cre (control) and Lkb1 hom KO mice. The mean discharge frequency in hypercapnia was recorded from the final minute of exposure after a relatively stable steady state discharge frequency had been established. Characteristic example traces of single/few fibre chemoafferent responses to hypercapnia for each group are shown in Figure 4.5A. Consistent with Section 4.2.2, the chemoafferent discharge frequency recorded under basal conditions was markedly reduced in the Lkb1 hom KO group compared with TH-Cre controls, measuring 0.22 ± 0.07 Hz and 0.54 ± 0.12 Hz respectively (Figure 4.5B). The absolute steady state discharge frequency detected during hypercapnic stimulation was also depleted in CBs isolated from Lkb1 hom KO mice (0.47 ± 0.18 Hz) in comparison with the TH-Cre controls (2.13 ± 0.33 Hz)(Figure 4.5B). Accordingly the CO_2 sensitivity (calculated as Δ discharge frequency (Hz) / mmHg PCO_2) was significantly depressed in Lkb1 hom KO CBs measuring only approximately 15% of that calculated for TH-Cre control group (Figure 4.5C). Lkb1 depletion appears not only to diminish the CB

response to hypoxia, but also the chemoafferent response to hypercapnia. These findings are therefore consistent with the idea that hypoxic and hypercapnic stimuli are exquisitively interdependent.

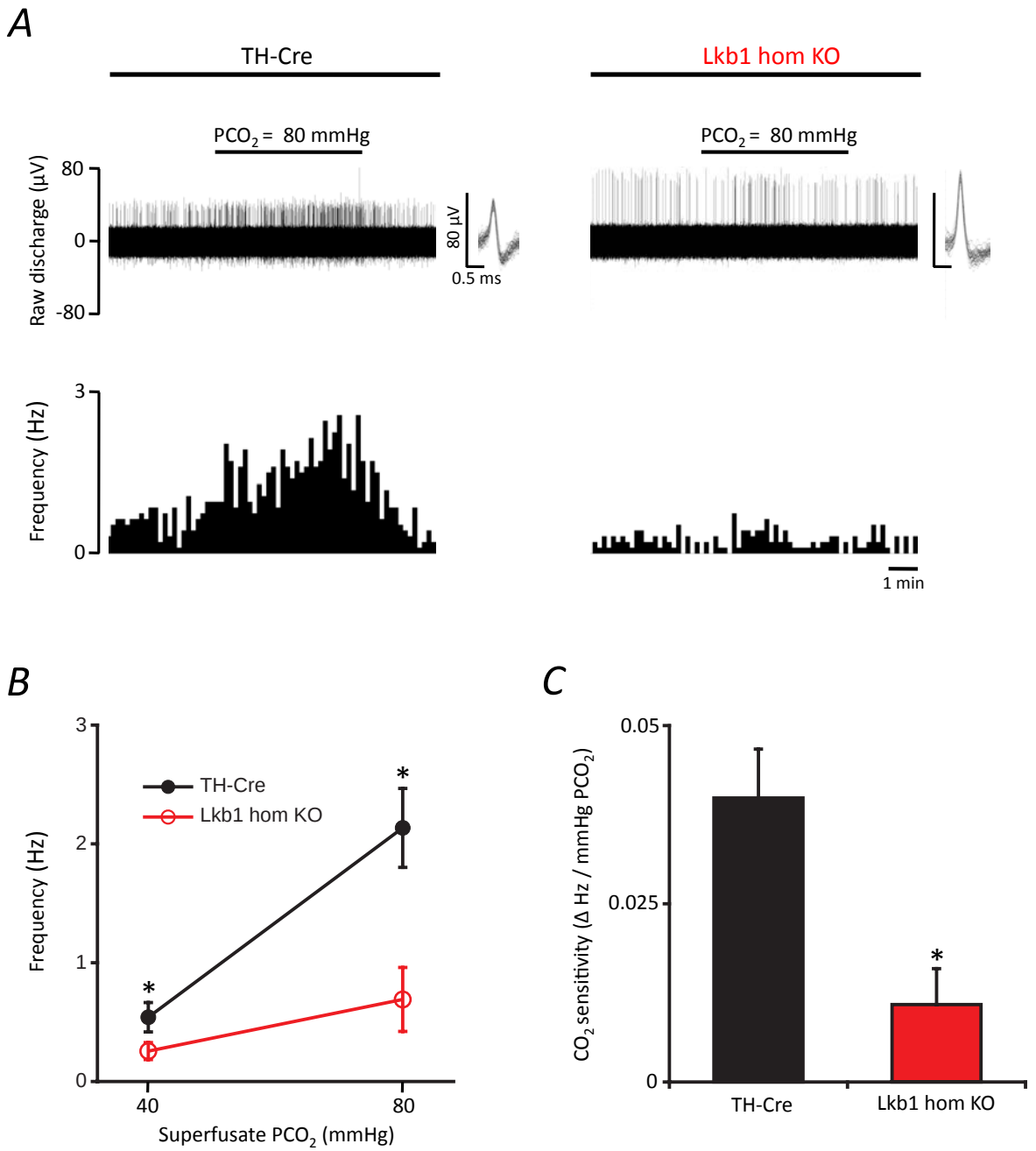


Figure 4.5 Lkb1 is an important mediator of the carotid body response to hypercapnia.
A) Example raw traces of single/few fibre chemoafferent recordings during exposure to hypercapnia for TH-Cre (control) and Lkb1 hom KO mouse groups. Raw discharge is shown (upper) along with frequency-time histograms that collated single fibre action potentials over 10 s intervals (lower). Single fibre discriminations are shown inset. B) Mean discharge frequencies recorded under normocapnic (PCO_2 ~ 40 mmHg) and hypercapnic (PCO_2 ~ 80 mmHg) conditions for TH-Cre (control); n=6 fibres from 4 mice, and Lkb1 hom KO; n=4 fibres from 4 mice. Error bars indicate Mean ± S.E.M. * denotes P < 0.05 TH-Cre v Lkb1 hom KO; one way factorial ANOVA with Bonferroni post hoc analysis. C) Calculated mean CO_2 sensitivity (Δ Hz / mmHg PCO_2) for TH-Cre and Lkb1 hom KO mouse groups. Error bars indicate Mean + S.E.M. * denotes P < 0.05 compared with TH-Cre; unpaired t-test.

4.3 Chapter synopsis and discussion

4.3.1 Summary of key findings

The main findings of the current chapter are described as follows;

- A reduction in Lkb1 protein expression significantly diminished basal CB chemoafferent activity.

- Lkb1 deletion virtually abolished the functional CB chemoafferent response to hypoxia.

- Depletion in Lkb1 also significantly suppressed the magnitude of CB excitation induced by hypercapnic stimulation.

4.3.2 Lkb1 expression in the carotid body is central to the generation of basal chemoafferent discharge frequency

Findings from the current study showed that a reduction in Lkb1 expression significantly depressed basal CB chemoafferent activity. This suggests that tonic Lkb1-AMPK signalling is necessary for the generation of a large component of the basal CSN discharge frequency.

The mechanism underpinning the precise origin of post-synaptic action potential production in the absence of any external stimulus is not known. Some reports indicate that type I cells spontaneously depolarise in normoxia/hyperoxia as a consequence of 5-HT mediated PKC activation and transient attenuation of the background K^+ current (277, 278). This in turn would evoke Ca^{2+} influx and neurotransmitter release leading directly to post-synaptic action potential generation.

A similar mechanism involving Lkb1-AMPK may also contribute to these spontaneous type I cell depolarisations. A number of specific AMPK phosphorylation targets in the type I cell have been characterised to date including the TASK, TREK-1 and BK_{Ca} channels (29, 54, 88). Of these, the TASK and TREK-1 channels would be expected to be active at the type I cell resting membrane potential. In the presence of Lkb1, a degree of AMPK activation even in normoxia/hyperoxia would potentially allow for some direct phosphorylation and inhibition of TASK and TREK-1 channel activity. This would reduce the background K^+ current and cause a transient type I cell depolarisation before the channel(s) was subsequently dephosphorylated. Future studies may focus on whether the overall phosphorylation status of TASK and TREK-1 channels dependent on PKC, AMPK and potentially PKA (see Chapter 6) activity may specifically determine

the rate and magnitude of spontaneous cellular depolarisations and regulate the basal chemoafferent discharge frequency. A mechanism of this nature may also act to establish the sensitivity of the type I cell to a range of physiological or pathological stimuli.

Identification of Lkb1-AMPK signalling in mediating basal CB activity may be of significant clinical importance. Elevations in basal CSN discharge frequency (termed sensory long term facilitation; sLTF) have been associated with an increase in sympathetic outflow and hypertension in animals and humans following periods of chronic intermittent hypoxia (2, 310). Interestingly, the sLTF following CIH has been proposed as being a consequence of higher rates of ROS production and inhibition of mitochondrial complex I activity (313). A sufficient depression of mitochondrial energy metabolism, even in normoxia, has the potential to increase the AMP:ATP ratio and further activate AMPK. Preliminary findings in Chapter 3 demonstrated that moderate chemostimulation (comparable to that seen in sLTF) induced by NO_2^- (a mild mitochondrial inhibitor) was partially reversed by pharmacological inhibition of AMPK. It may be plausible to suggest that the CB sLTF following CIH is a direct consequence of an increase in Lkb1-AMPK signalling following mild ROS induced mitochondrial inhibition. This should be examined directly in future work. Selective inhibitory targeting of the Lkb1-AMPK pathway in the CB may prove to be clinically important in reducing the sLTF and hypertension in patients with sleep disordered breathing.

4.3.3 Lkb1 provides the key to carotid body activation by hypoxia

The experiments performed in Chapter 3 showed that mild mitochondrial inhibition enhanced the CB hypoxic sensitivity. This provided further evidence emphasising that

mitochondrial inhibition is the key to CB O_2 sensing. The identification of a clearly defined signalling cascade that links the run-down in mitochondrial energy metabolism with type I cell stimulation has remained elusive. The principal focus of the current chapter was to investigate if activation of the Lkb1-AMPK pathway was the critical intermediate step in mediating this process.

Partial or complete Lkb1 deletion significantly diminished the functional CB chemoafferent response to hypoxia. The findings from the current study provide conclusive support for the proposal that Lkb1-AMPK signalling is necessary for full carotid body activation by hypoxia. It is suggested that the Lkb1-AMPK system acts as the primary sensor of a run-down in mitochondrial oxidative metabolism during hypoxia and is also the principal effector in mediating hypoxia stimulus response coupling within the type I cell.

Additional experiments were performed on isolated type I cells by colleagues at the University of Leeds and not by the author of this thesis. These results demonstrated that the elevation in $[Ca^{2+}]_i$ observed in response to hypoxia was almost completely abolished in type I cells isolated from Lkb1 hom KO mice (data not shown). It is proposed that the lack of a full chemoafferent response to hypoxia seen in the intact CB preparation was due to a depression in type I cell hypoxic sensitivity and not because of any functional deficit in the chemoafferent neurones.

An elevation in the AMP:ATP ratio and subsequent activation of AMPK in response to mitochondrial poisons and hypoxia is well characterised (5, 101). Lkb1 mediated phosphorylation of the Thr 172 residue on the α subunit of AMPK is recognised as the

essential process that activates AMPK in response to a number of pharmacological and physiological stimuli including hypoxia (105, 106, 346). It is proposed that the deletion of Lkb1 in these experiments prevented any hypoxia induced AMPK activation and subsequent phosphorylation of downstream targets and this accounted for a reduction in type I cell depolarisation, Ca^{2+} influx, neurosecretion and the whole organ chemoafferent response to hypoxia. The precise downstream AMPK phosphorylation targets in the CB type I cell include sites on the TASK, TREK-1 and BK_{Ca} channels (29, 54, 88), all of which are implicated in CB O_2 sensing.

CBs isolated from Lkb1 het KO and Lkb1 hom KO animals both showed the same statistical degree of response attenuation upon hypoxic stimulation. Measurements of type I cell Lkb1 protein expression for all groups were not made, but it is estimated that Lkb1 expression in the Lkb1 het KO CB was approximately 50% of the TH-Cre control. This may be an overestimate given that insertion of the loxP excision sequence has been shown to depress Lkb1 expression even in the absence of Cre recombinase (106). It still appears that depletion of CB Lkb1 expression in the Lkb1 het KO group by approximately 50% severely depressed the CB response to hypoxia, and further deletion in the Lkb1 hom KO group caused no further functional shortfall. This implies that AMPK-mediated activation of the CB response to hypoxia requires a minimum of 50% of the control level of Lkb1 protein expression.

Mean data from both the Lkb1 hom KO and Lkb1 het KO CB groups suggested that although the response to hypoxia was markedly attenuated, a very small residual component was still preserved. Type I cell Lkb1 protein expression analysis did not positively detect any Lkb1 mRNA expression in cells isolated from the Lkb1 hom KO

group. Therefore, the residual response is unlikely to be because of incomplete knock down of Lkb1. Rather, the small elevation in discharge frequency is most probably due to CB activation evoked independently of Lkb1-AMPK signalling. Given the link between mitochondrial electron transport and CB hypoxic chemotransduction highlighted in Chapter 3 it is suggested that some type I cell depolarisation may be a consequence of direct TASK-like channel deactivation following the absolute reduction in $[ATP]_i$, perhaps under conditions of very severe hypoxia (343). Even so, these data clearly emphasise that the vast majority of hypoxia stimulus response coupling in the CB is critically dependent on Lkb1-AMPK signalling.

4.3.4 Lkb1 deletion attenuates the carotid body response to hypercapnia; is this due to a complete lack of type I cell function?

Complete deletion of Lkb1 expression significantly diminished the CB chemoafferent response to hypercapnia. The reasons for this are unclear. It has been shown that the hypoxic sensitivity of the CB is augmented by hypercapnia, indicative of a strong interaction between these two stimuli (46). The observed absence of hypercapnic sensitivity in the Lkb1 hom KO group may have been a direct consequence of a diminished hypoxic sensitivity.

Translation of hypoxic or hypercapnic stimuli into a functional elevation in chemoafferent discharge frequency involves many of the same chemotransduction processes including inhibition of background TASK-like and BK_{Ca} conductances, type I cell depolarisation, Ca^{2+} influx and neurosecretion (18). Whilst there is little evidence for AMPK being stimulated directly by hypercapnia, the absence of basal Lkb1 mediated AMPK

activation may have altered the threshold required for hypercapnic response initiation by downregulating any or all of the processes described above.

Alternatively, the absence of responses to both hypoxia and hypercapnia may have been due to a complete loss of type I cell chemoreceptor function. The STRAD-Lkb1-MO25 complex is known to phosphorylate a total of 13 kinases, all of which are part of the AMPK sub-family (348). At present very little is known about the function of these proteins but it has been suggested that some may be involved in regulation of cell growth, proliferation and polarity (reviewed in (349)). It is possible that the loss of Lkb1 disrupts the full development of important type I cell specific chemotransduction processes.

That said, experiments performed on isolated type I cells by colleagues at the University of Leeds demonstrated that Lkb1 deficient cells had an augmentation in $[Ca^{2+}]_i$ in response to a 60 mM K^+ external stimulus (albeit seemingly less than those rises recorded for TH-Cre control type I cells) (data not shown). In addition, a small degree of chemoafferent excitation was observed in almost all Lkb1 hom KO CBs in very severe hypoxic conditions and all fibres generated a measurable level of basal discharge. This indicates that some of the key processes involved in type I cell hypoxic and hypercapnic chemotransduction are maintained even in the complete absence of Lkb1. Future studies should, however, be performed to demonstrate that the isolated CBs from the Lkb1 hom KO mice are capable of generating robust responses to stimuli that are unaffected by the loss of hypoxic sensitivity. This could be achieved by administration of exogenous doxapram (TASK channel inhibitor) (350), a substance that would be

predicted to depolarise the type I cell directly and elevate chemoafferent discharge frequency independent of Lkb1-AMPK activation (351).

The full impact of Lkb1 deletion on type I cell phenotype is unknown. Although the results presented in this chapter are strongly indicative of Lkb1-AMPK signalling being central to the CB hypoxia stimulus response coupling process, they should still be viewed with a significant degree of caution given the other uncharacterised potential effects that partial or total Lkb1 deletion may have on type I cell function or viability.

4.3.5 Conclusions

In the CB, Lkb1 is required for the generation of the majority of the basal chemoafferent discharge frequency. Furthermore, Lkb1 is essential for evoking the full chemoafferent response to hypoxia. This suggests that, even in normoxia, Lkb1 phosphorylates AMPK, thus provoking a degree of basal AMPK activation. Importantly, increased phosphorylation of AMPK by Lkb1 is necessary to further activate the CB and to enhance the chemoafferent discharge frequency in hypoxia. These data support the proposal that Lkb1-AMPK signalling provides the critical link between hypoxia induced suppression of mitochondrial energy metabolism and type I cell stimulation.

5. Studying the effects of glucose deprivation on the intact carotid body *in vitro*: low glucose sensing or metabolic depletion?

5.1 Chapter introduction and overview

A number of previous studies have proposed that the CB is sensitive to low glucose and functions as a physiological glucose receptor within the whole animal (238, 246, 248). Given the strong association between metabolic stress and CB stimulation in hypoxia described in the previous two chapters, it is possible that a similar mechanism leading to CB excitation may exist in response to a fall in glucose concentration. If a reduction in substrate delivery into the mitochondria leads to the depression of ATP synthesis then, as with hypoxia, this may lead to activation of Lkb1-AMPK. Accordingly, central low glucose sensing neurones are recognised as being stimulated subsequent to an increase in AMPK activity (235, 236).

Whilst a role for the mammalian CB in glucose homeostasis has been clearly defined (see Chapter 1), there remains a debate as to whether the CB responds either directly and rapidly to low glucose, or indirectly to some other blood-borne stimulus released as a consequence of systemic hypoglycaemia.

The direct impact of low glucose on CB function appears equivocal. Freshly isolated intact CB preparations seem to be acutely unresponsive to low glucose even at sub-

physiological concentrations. Kumar and colleagues demonstrated that the *in vitro* CB chemoafferent activity was not enhanced upon exposure to 2 mM glucose (242) and this same glucose concentration reduced the CB sensitivity to hypercapnia (243). Conde et al. observed that complete glucose deprivation did not acutely stimulate whole CB ^{3}H-CA or ATP release and failed to potentiate the CB response to mild (7% O_2) hypoxia (247). However, sustained exposure to glucose deprivation has been shown to stimulate the intact CB, with an augmented secretion of neurotransmitters being observed after approximately 40 minutes (247).

In contrast, long-term (> 24 hours) *in vitro* CB culture preparations show robust and acute sensitivity to physiological levels of low glucose. In the CB slice preparation, 3.3 mM glucose inhibited type I cell K^+ conductance and evoked a rapid and measurable elevation in DA release (246). Intracellular voltage recordings of petrosal neurones co-cultured with type I cell clusters demonstrated that 3.3 mM glucose almost instantaneously enhanced the action potential firing frequency, indicative of intrinsic type I cell low glucose sensitivity (248). In both instances, responses to physiological levels of low glucose were only detected when the superfusate PO_2 was lowered to 90 mmHg, demonstrating a PO_2 dependence on the CB low glucose sensitivity. Zhang et al suggested that the absence of a response in the intact CB, observed in other studies, was due to the high level of PO_2 used in these experiments (248). Given that hyperoxia attenuates the response to hypercapnia (46), it is notional that hyperoxia may act to impair responses to low glucose in a similar way. In the intact CB explant model, following 24 hours of whole organ culture, complete removal of glucose from the superfusate did not increase ^{3}H-CA release in 20% O_2 but it did potentiate the response to hypoxia, again emphasising that low glucose sensitivity is dependent on PO_2 (249).

The focus of the following set of experiments was as follows:

1. To establish if the nature of the response of the freshly isolated intact carotid body *in vitro* to prolonged glucose deprivation is a consequence of direct low glucose sensing or time dependent metabolic reserve depletion.

2. To examine if there is a dependence of PO_2 on the excitation evoked by glucose deprivation in the freshly isolated CB.

3. To investigate whether the conditions of CB tissue incubation, following isolation, altered the sensitivity of the whole organ to subsequent low glucose stimulation.

5.2 Results

5.2.1 The impact of basal chemoafferent fibre discharge frequency on the time taken for the carotid body to respond to glucose deprivation

Recordings of single unit chemoafferent discharge frequency made in the rat *in vivo* have shown a frequency of approximately 0.25–1.5 Hz in arterial normoxia (41). In the first experiments in the present study, the superfusate PO_2 was adjusted (at a fixed PCO_2 of approximately 40 mmHg) to set a basal frequency of 0.25–1.5 Hz in 11 mM glucose consistent with those observations made *in vivo* in arterial normoxia. After this baseline level of discharge frequency was established the CB was exposed to a glucose free superfusate (osmolality balanced with mannitol) equilibrated at the equivalent PO_2. The time taken to respond to the glucose free stimulus was taken when a level of discharge 10% or greater than the mean control basal discharge frequency had been achieved and maintained for at least 30 seconds.

An example of a characteristic response to glucose deprivation at a basal level of discharge between 0.25–1.5 Hz is shown in the upper panel of Figure 5.1A. All fibres set at a basal level of discharge frequency between 0.25–1.5 Hz, did not respond acutely to glucose deprivation, consistent with a lack of intrinsic low glucose sensitivity. At this level of basal discharge frequency an increase in discharge frequency was detected only after at least 20 minutes of glucose free exposure. After a short period of measured excitation, peaking at 3.73 ± 0.90 Hz, the discharge frequency fell to below basal levels or was completely abolished.

The delayed response to glucose free exposure observed after 20 minutes or more was hypothesised as being a consequence of time dependent depletion in metabolic reserve. In the next series of experiments, the superfusate PO_2 was decreased so that the basal chemoafferent discharge frequency increased to levels consistent with moderate hypoxia (see Chapter 2). This was predicted to elevate the rate of glucose utilisation through increased glycolysis (352). After a steady state basal frequency had been established (that was still significantly below the level of a peak hypoxic response) glucose was removed from the superfusate.

Example traces of the effect of an increase in basal frequency on the response time to glucose deprivation are shown in the middle and lower panels of Figure 5.1A. Regression analysis of the grouped data showed a strong correlation between the level of basal chemoafferent fibre discharge frequency and response time to glucose deprivation ($R^2 = 0.7$, $P < 0.05$) (Figure 5.1B). The stimulation induced by glucose deprivation was still not instantaneous even at very high basal frequencies (Figure 5.1B). These results are consistent with the notion that the basal rate of glycolysis determines the time taken for the CB to respond to glucose deprivation and that a moderate level of steady state hypoxia does not induce any acute low glucose sensitivity.

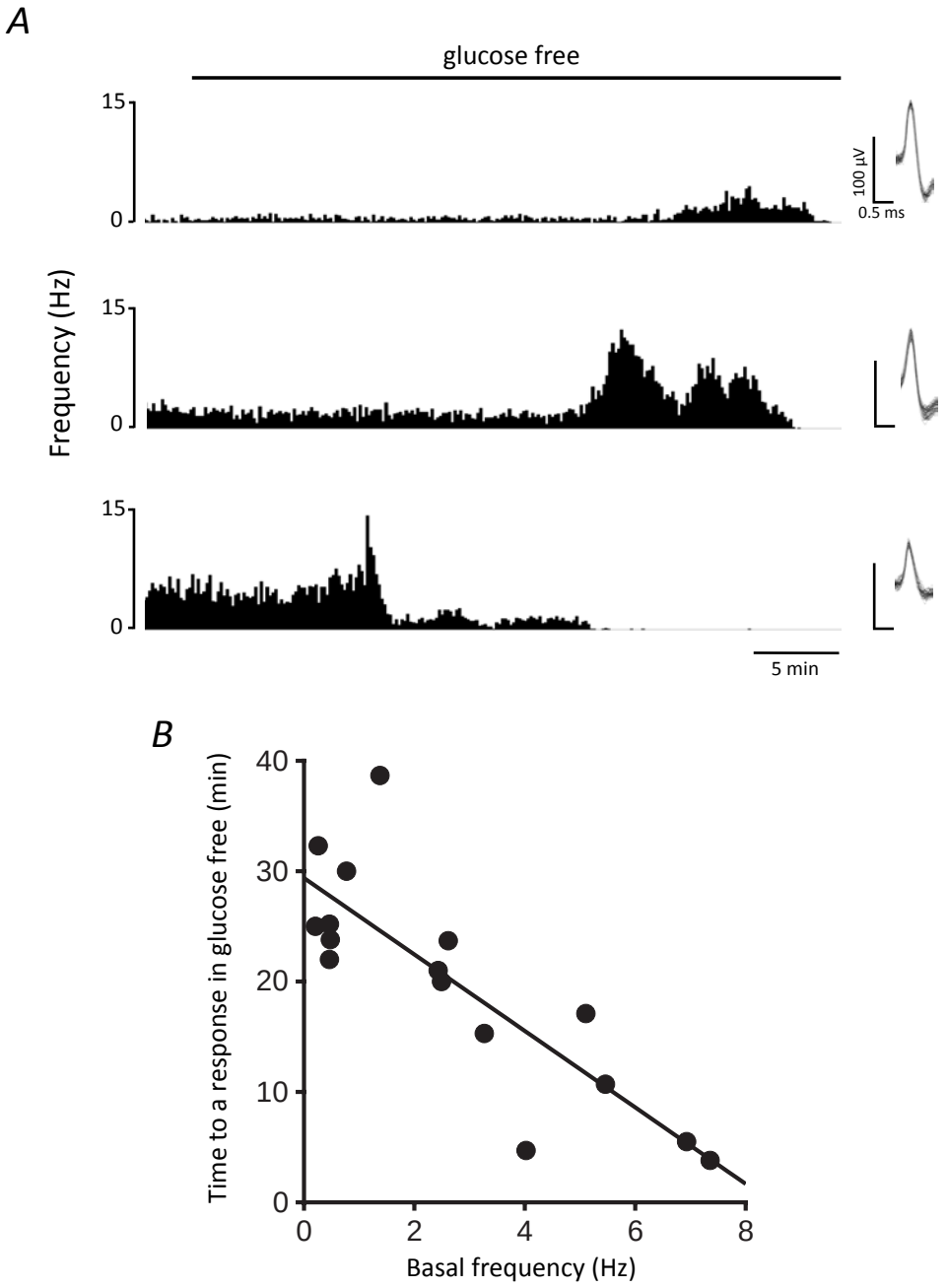


Figure 5.1 The time taken to respond to glucose deprivation is dependent on basal chemoafferent activity.
The data presented was taken from 16 single chemoafferent fibres from 13 CB preparations. A) Example traces showing the responses to glucose deprivation with increasing initial basal discharge frequencies (upper to lower). Chemoafferent single fibre raw discharge was recorded and expressed in frequency histograms binned at 10 second intervals. For each trace multiple action potentials have been overdrawn to show the single fibre discrimination. B) Grouped data showing a correlation between basal frequency and the time taken to respond to glucose deprivation ($R^2 = 0.7$, $P < 0.05$; linear regression analysis).

5.2.2 Exposure of the carotid body to severe hypoxia reduces the time taken to respond to subsequent glucose deprivation

The level of glucose utilisation through glycolysis in the CB is known to be dependent on the severity of hypoxia (352). It was next examined whether stimulation of the CB by severe hypoxia had the potential to reduce the time taken to respond to a subsequent glucose free stimulus; potentially through depletion of any metabolic reserve stored within either the type I cell clusters or the afferent neurones. The CB was exposed to one or two 4-5 minute periods of severe hypoxia (superfusate PO_2 approximately 60 mmHg) in 11 mM glucose. Subsequently, after a steady state level of discharge frequency had been re-established (between 0.25–1.5 Hz), superfusate glucose was removed and the time taken to a response was measured. An example of the characteristic response to glucose deprivation following two exposures to severe hypoxia is shown in Figure 5.2A. Control experiments were performed on CBs that were not subjected to hypoxia before removal of glucose. This control group was the same as described in 5.2.1.

A single episode of severe hypoxia decreased the time taken to respond to subsequent glucose deprivation by approximately 40% and a greater reduction in time of 65% was observed following two hypoxic exposures (Figure 5.2B). The peak frequency response to glucose deprivation was not significantly different among the three groups (3.73 ± 0.90 Hz; control, 2.83 ± 0.35 Hz; post one hypoxic exposure, 4.02 ± 0.53 Hz; post two hypoxic exposures). This verified that prior exposure to severe hypoxia did not attenuate the magnitude of the chemoexcitation evoked by glucose deprivation but did lessen the time taken to respond.

In all experiments where severe hypoxia was applied before the glucose free stimulus, the maximum hypoxic frequency was initially recorded (Figure 5.2A). The peak chemoafferent discharge frequency was also measured in glucose free conditions before it diminished. Examples of the responses of the same fibre to glucose deprivation and hypoxia are shown in Figure 5.3A. Comparison of the mean peak frequencies attained in severe hypoxia and glucose deprivation identified that the degree of chemostimulation induced by glucose deprivation was less than 15% of that observed during severe hypoxia (Figure 5.3B).

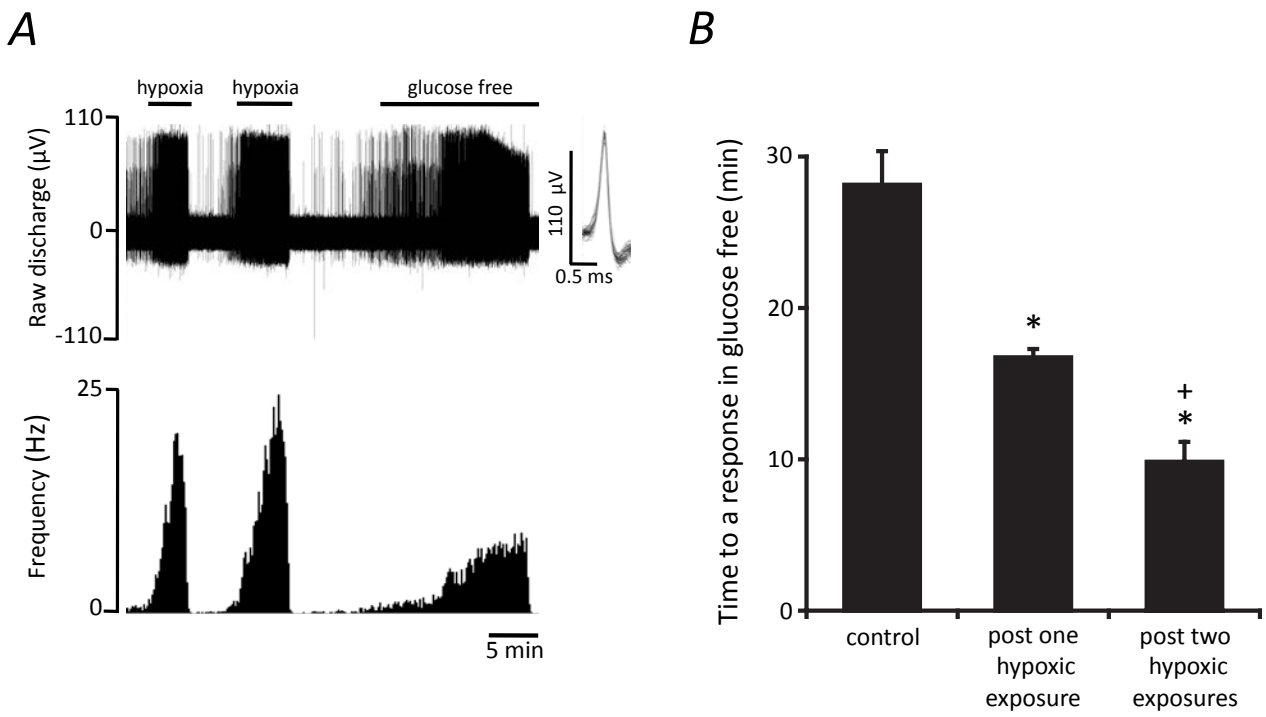


Figure 5.2 The time taken to respond to glucose deprivation is significantly reduced by prior exposure to severe hypoxia.
The data presented was taken from 7 single chemoafferent fibres from 6 CB preparations in the control group, 9 fibres from 5 CB preparations in the post one hypoxic exposure group and 12 fibres from 8 CB preparations in the post two hypoxic exposures group. A) Example trace showing the response to a glucose free stimulus following two 4-5 minute episodes of severe hypoxia. Raw neuronal discharge is shown in the upper panel and discharge from a single fibre is expressed in frequency histograms binned at 10 second intervals below. Inset; Multiple action potentials have been overdrawn to show the single fibre discrimination. B) Grouped data showing that one or two exposures to severe hypoxia significantly decreased the time taken to respond to subsequent glucose deprivation. Error bars indicate + SEM.
* denotes a significance of $P < 0.05$ compared with the time to a response to glucose free in the control group and + denotes a significance of $P < 0.05$ compared with the time taken to a response to glucose free in the post single hypoxic episode group; one way factorial ANOVA with Bonferroni post hoc analysis.

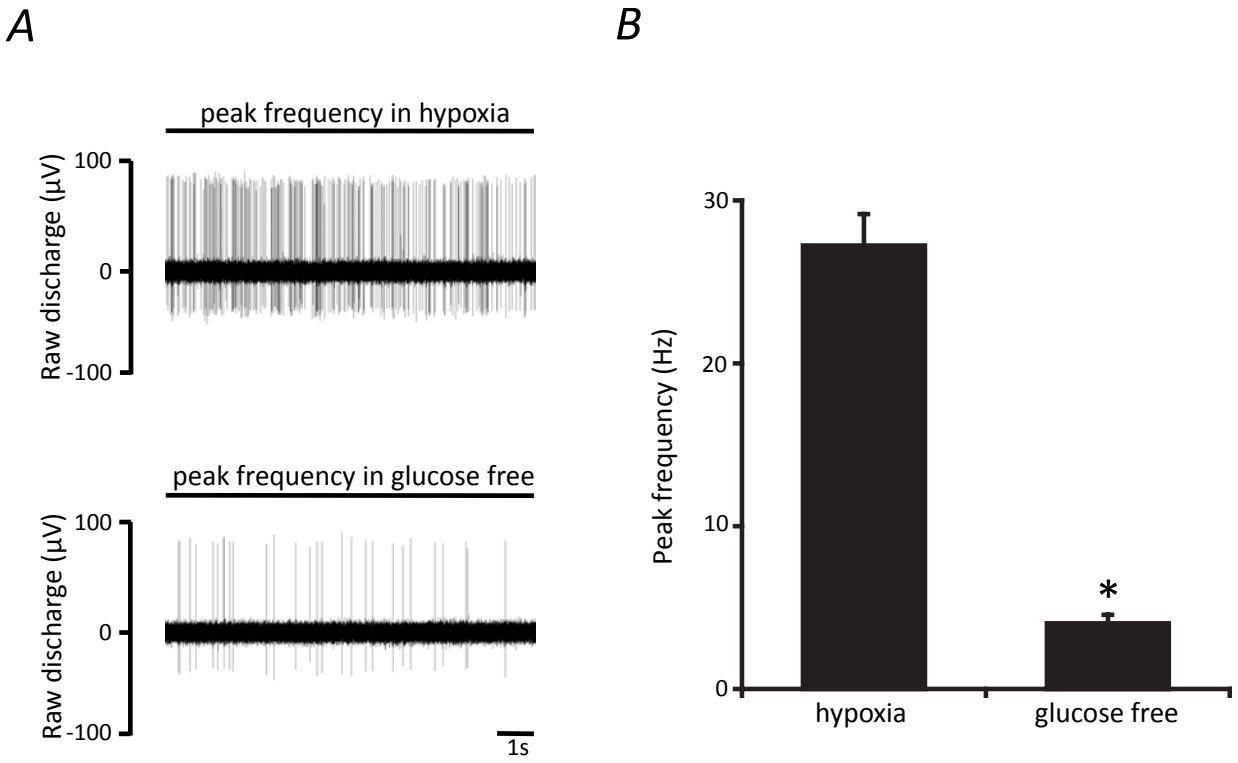


Figure 5.3 The amplitude of a chemoafferent excitation induced by glucose deprivation is significantly less than that evoked by severe hypoxia.
A) Example of the raw discharge taken from the same single fibre showing a 10 second period of maximal hypoxic (upper) and glucose free (lower) stimulation. B) Grouped data demonstrating that the response to glucose deprivation was significantly smaller than that induced by hypoxia. The mean data was taken from 12 fibres from 8 CB preparations that were exposed to both severe hypoxia and glucose deprivation. Error bars indicate + SEM. * denotes a significance of $P < 0.05$ compared with hypoxia; paired t-test.

5.2.3 The lack of acute excitation induced by glucose deprivation in the carotid body; a functional role for glycogen

Failure of glucose deprivation to acutely stimulate the CB would indicate that type I cells have a level of metabolic reserve that can be utilised in the event of complete absence of extracellular substrate delivery. The most common form of metabolic reserve in neuronal brain tissue is glycogen, stored predominately in the neighbouring astrocytes (353). Glycogen is metabolised in astrocytes to form glucose-6-phosphate, which in turn can be converted to lactate and released into the interstitium (354, 355). Uptake of lactate by the adjacent neurones supports their metabolism in times of increased activity (356, 357). Very recently, glycogen metabolism to form lactate has also been implicated in supporting the function of peripheral nerves during glucose free exposure (358). To this point a functional role for glycogen in the CB has not been defined.

Immunohistochemical staining of 10 μm sections of CB tissue showed co-localisation of tyrosine hydroxylase (TH) (a classical type I cell marker) with both glycogen synthase I and the brain/neuronal isoform of glycogen phosphorylase BB (Figure 5.4A and B respectively). In both instances, staining was reproducible in a total of 3 sections from 3 different CBs. In all sections staining for glycogen synthase I and glycogen phosphorylase BB was not completely confined to TH positive type I cells suggesting a presence of these enzymes in other non TH containing cells e.g. chemoafferent neurones or possibly type II cells (Figure 5.4A and B). Addition of the secondary antibodies without prior primary antibody staining was performed to examine potential non-specific secondary antibody binding. In these instances fluorescence intensity was almost completely abolished. These data indicate that type I cells along with other cells in the CB contained enzymes capable of both synthesis and metabolism of glycogen.

To study the functional importance of glycogen in maintaining CB activity during glucose deprivation, experiments were performed on CB tissue exposed to the glycogen phosphorylase inhibitor 1, 4-Dideoxy-1, 4-imino D-arabinitol hydrochloride (DAB); an agent shown to attenuate glycogenolysis in isolated rat hepatocytes (359). A DAB concentration of 100 μM was chosen (five times that used on single cells), in order to overcome any diffusion limitations present within the intact CB preparation. 100 μM DAB was initially added during superfusion with 11 mM glucose and the same DAB concentration was maintained during glucose free exposure. An example response to glucose deprivation in the presence of DAB is shown in Figure 5.5A.

Partial or complete inhibition of glycogen metabolism with DAB significantly reduced the time taken to respond to glucose free exposure by approximately 33% (Figure 5.5B). In all experiments, initial basal discharge was established between 0.25–1.5 Hz before removal of glucose. Maximal stimulation evoked by glucose deprivation was not significantly different between groups; control 5.1 ± 1.2 Hz and DAB 3.3 ± 0.7 Hz (unpaired t-test). These data support the proposal that glycogen metabolism has an important role in supporting the chemoafferent discharge frequency during glucose deprivation.

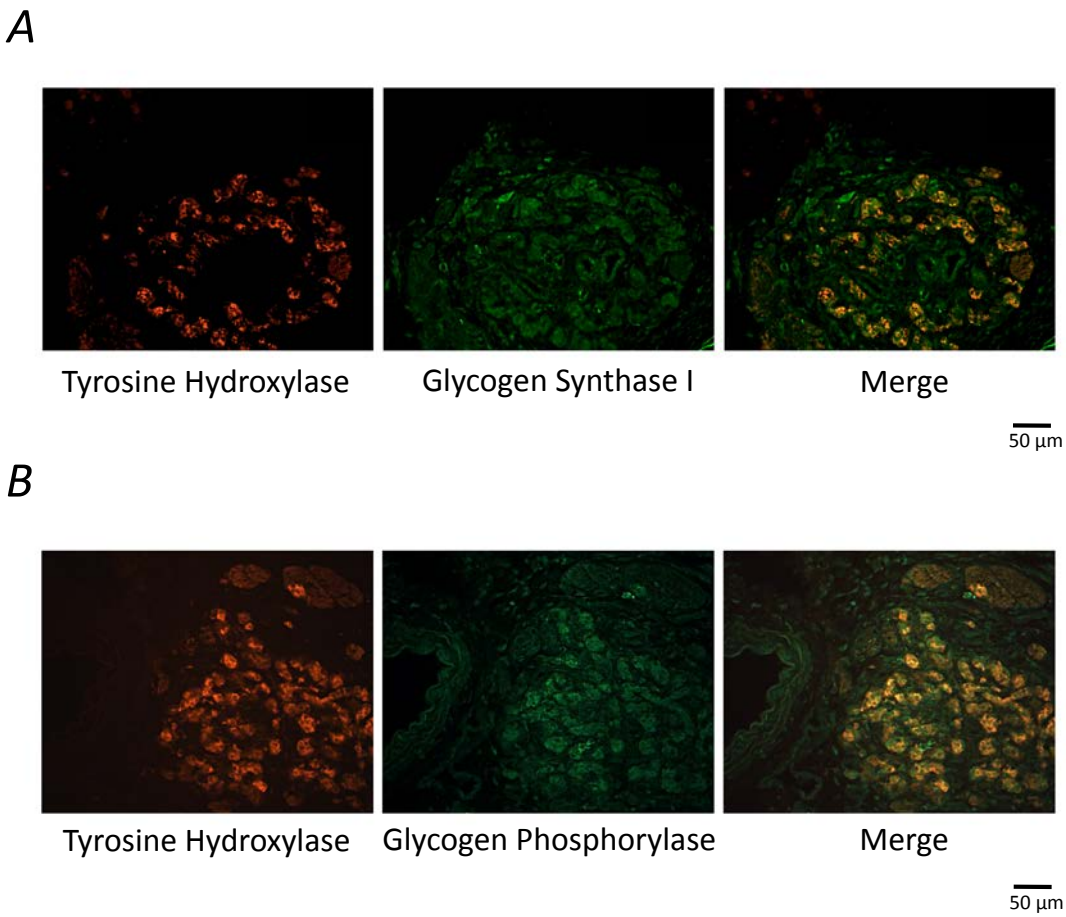


Figure 5.4 Co-localisation of tyrosine hydroxylase with glycogen synthase I and neuronal glycogen phosphorylase in the rat carotid body.
CB sections (10 µm) were stained with antibodies specific to glycogen synthase I (A) and neuronal glycogen phosphorylase (B) along with tyrosine hydroxylase (TH), a marker for type I cells. Merged images are shown in the right hand panels suggesting significant co-localisation. (Scale bar : 50 µm).

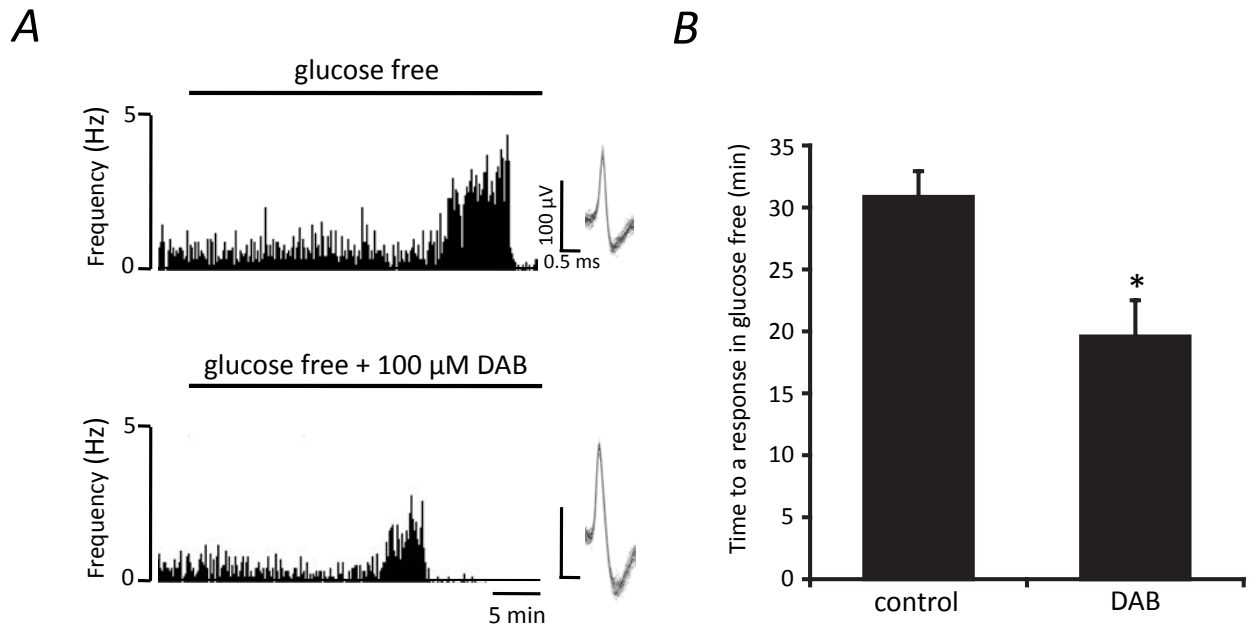


Figure 5.5 Glycogen is significant in maintaining chemoafferent discharge frequency during glucose deprivation.
Glycogen reserve was inhibited using a known blocker of glycogen phosphorylase, 4-Dideoxy-1, 4-imino D-arabinitol hydrochloride (DAB). Data presented is taken from 8 fibres from 8 CB preparations (control), and 6 fibres from 4 CB preparations (DAB). A) Example traces showing the response to glucose deprivation are demonstrated in control CBs and in CBs treated with DAB. Chemoafferent single fibre raw discharge was recorded and expressed in frequency histograms binned at 10 second intervals. For each trace multiple action potentials have been overdrawn to show single fibre discrimination. B) Grouped data showing that DAB significantly decreased the time taken to respond to glucose deprivation. Error bars indicate + SEM, * denotes a significance level of $P < 0.05$ compared with the time to a response in the control group; unpaired t-test.

5.2.4 Stimulation evoked by glucose deprivation can be reversed by 1 mM glucose; evidence against the carotid body being a physiological glucose receptor

For the CB to be considered a direct physiological gluco-sensitive tissue it would be expected to show a degree of stimulation to concentrations of low glucose within the physiological range. Two studies have reported acute CB tissue stimulation induced by physiological low glucose concentrations *in vitro* in the CB slice and the CB co-culture preparations respectively (246, 248). However, an acute response to physiological low glucose has not been observed in the intact CB preparations monitoring either chemoafferent discharge frequency or ^{3}H-CA release (242, 247).

Experiments performed in all previous sections of this chapter have identified a response to prolonged glucose deprivation. In this section, it was examined if this response was maintained by adding back just 1 mM glucose, as would be predicted if the CB tissue was a functional low glucose sensor. CBs were first exposed to a single 5 minute period of severe hypoxia in order to reduce the time taken for them to respond to subsequent glucose deprivation (see Section 5.2.2). A baseline discharge frequency between 0.25–1.5 Hz was established and the same PO_2 was sustained for the remainder of the experiment. During the initial glucose free evoked stimulation 1 mM glucose was returned to the superfusate. A second glucose free stimulus was then maintained for the duration of the protocol. A characteristic example is shown in Figure 5.6A. In all experiments the chemoexcitation generated by glucose deprivation was rapidly (within seconds) reversed by the addition of only 1 mM glucose (Figure 5.6B). Chemoafferent stimulation to a second glucose free stimulus was observed that was equal or greater in magnitude compared with the initial response indicating that the

decline in discharge frequency evoked by 1 mM glucose was not due to CB failure or neurotransmitter depletion (Figure 5.6A and B).

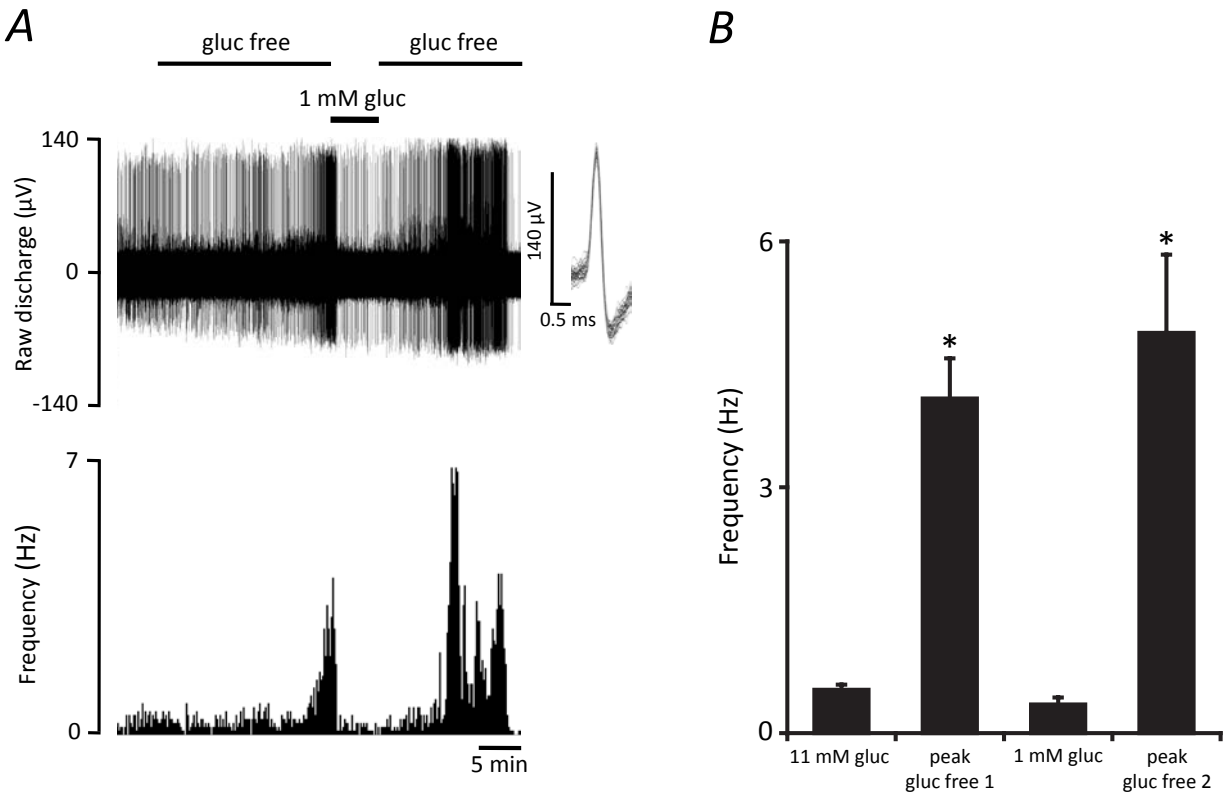


Figure 5.6 Stimulation evoked by glucose deprivation is completely reversed by 1 mM glucose. A) Example trace showing the effect of 1 mM glucose on the response to complete glucose deprivation. Raw neuronal discharge is shown in the upper panel and discharge from a single fibre is expressed in frequency histograms binned at 10 second intervals below. Inset; Multiple action potentials have been overdrawn to show the single fibre discrimination. B) Grouped data showing that addition of 1 mM glucose abolishes the response to initial glucose free stimulation. Data presented was taken from 6 spikes from 3 CB preparations. Error bars indicate + SEM. * denotes a significance of $P < 0.05$ compared with the basal frequency in 11 mM glucose; one way repeated measures ANOVA with Dunnett's post-hoc comparisons test.

5.2.5 Excitation induced by glucose deprivation can be abolished or is rapidly reversed by lactate and pyruvate; evidence for a response to metabolic stress rather than directly to low glucose

Prolonged exposure of the CB to glucose deprivation would be expected to reduce the rate of glycolysis following total depletion of any glycogen reserve. In the absence of other substrates entering the TCA cycle or electron transport chain this would subsequently decrease cytosolic and mitochondrial NADH levels, causing a reduction in electron flux and decreased ATP production.

Studies in this section were designed to investigate if prolonged glucose deprivation stimulated chemoafferent excitation through a reduction in glycolysis and cellular energy status. CBs were exposed to a glucose free superfusate with or without the addition of 5 mM lactate and 0.5 mM pyruvate. Lactate and pyruvate concentrations were selected to maintain a standard lactate: pyruvate ratio of approximately 10:1 (360). The PO_2 was adjusted to set a basal level of chemoafferent discharge frequency between 0.25–1.5 Hz and remained the same throughout the protocol.

Examples of the chemoafferent discharge frequency measured from two individual preparations during glucose free exposure with and without lactate and pyruvate are shown in Figure 5.7A. An increase in mean discharge frequency was detected in the glucose free only group at 30, 35 and 40 minutes (Figure 5.7B). In contrast no chemoafferent stimulation was detected in the presence of lactate and pyruvate (Figure 5.7B). This suggests that stimulation during glucose deprivation is a consequence of a time dependent run-down in energy status.

However, sustained incubation of the CB tissue in lactate and pyruvate may have encouraged generation of *de novo* glucose through the gluconeogenesis pathway. An absence of a response therefore may have been due to the maintenance of intracellular glucose concentration generated from the lactate and pyruvate substrates.

Investigations subsequently focused on whether the potential metabolic stress and chemostimulation during prolonged glucose deprivation could be acutely reversed by addition of lactate and pyruvate. During the first glucose free induced response 5 mM lactate and 0.5 mM pyruvate were added to the superfusate. An example of the impact of lactate and pyruvate on the chemoafferent discharge frequency recorded from a single fibre at the point of glucose free induced stimulation is shown in Figure 5.7C. In all fibres studied, addition of lactate and pyruvate rapidly restored the enhanced chemoafferent discharge frequency to basal levels within seconds (5.7C and D). Because of the immediate nature of the decline in chemoafferent activity evoked by addition of these substrates it was proposed that they bypassed the glycolysis pathway and entered the TCA cycle independent of *de novo* glucose synthesis through gluconeogenesis. This is consistent with the chemostimulation caused by prolonged glucose deprivation being purely dependent on a complete run-down in glycolysis.

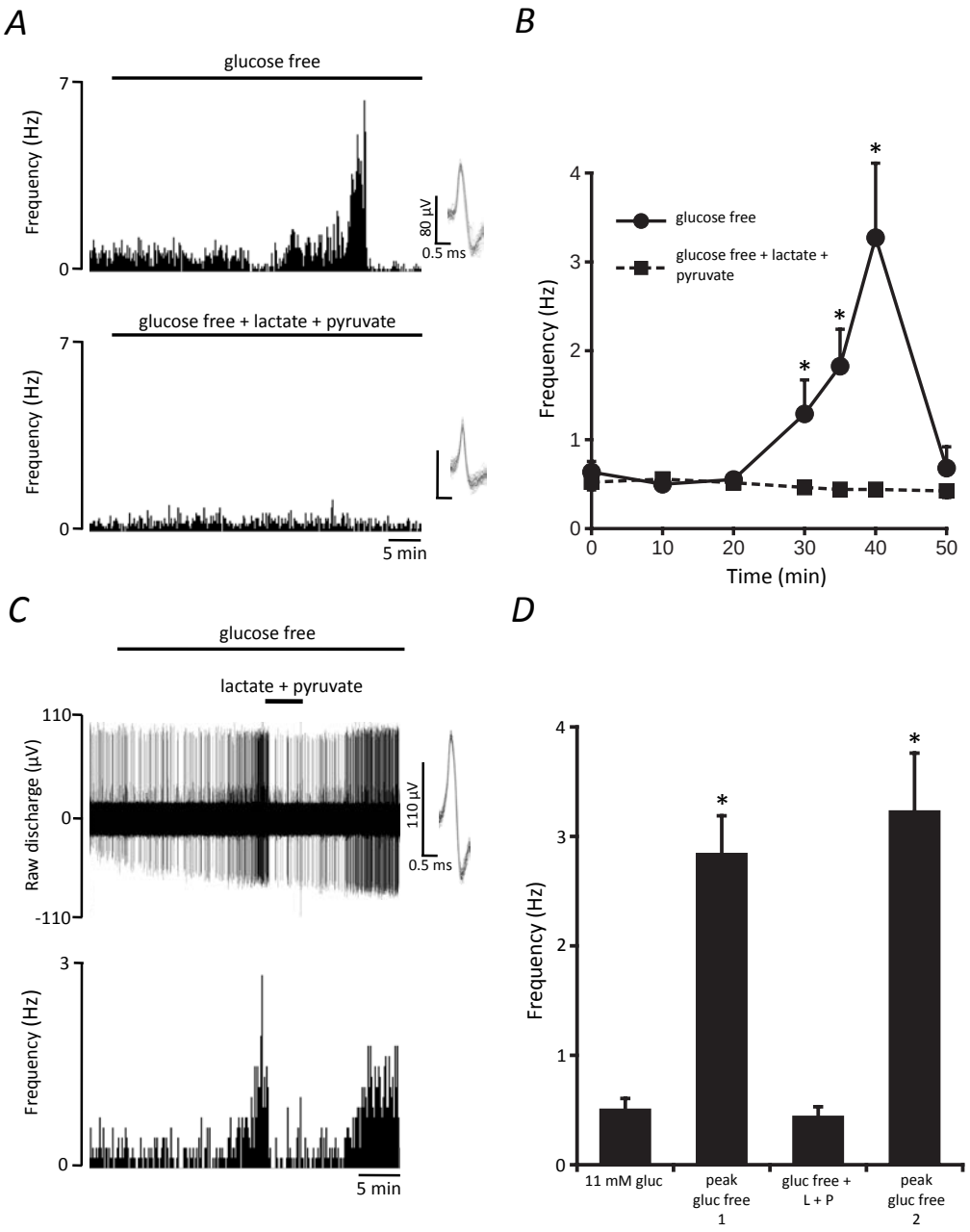


Figure 5.7 Chemoexcitation evoked by glucose deprivation can be abolished or is rapidly reversed by lactate and pyruvate.
A) Individual traces showing the chemoafferent discharge frequency in the glucose free with and without 5 mM lactate and 0.5 mM pyruvate, upper and lower panels respectively. Discharge frequency is expressed in 10 second interval histograms. Single fibre discrimination is shown inset. B) Mean discharge frequencies calculated at time points during exposure to glucose deprivation in the presence or absence of 5 mM lactate and 0.5 mM pyruvate. Data is presented from 8 fibres from 8 CB preparations (glucose free only) and 8 fibres from 6 CB preparations glucose free plus lactate and pyruvate. Error bars indicate ± SEM, * denotes $P < 0.05$ compared with basal frequency; one way repeated measures ANOVA with Dunnett's post-hoc comparisons test. C) As in A) but with lactate and pyruvate rapidly reversing the initial stimulation evoked by glucose deprivation. Raw discharge frequency from a single fibre is demonstrated (upper) along with frequency histograms (lower). D) Mean data showing that the response to glucose deprivation is restored to basal levels by the addition of lactate and pyruvate. Data presented is from 9 fibres from 5 CB preparations. Error bars indicate + SEM, * denotes $P < 0.05$ compared with basal frequency in 11 mM glucose; one way repeated measures ANOVA with Dunnett's post-hoc comparisons test.

5.2.6 Evaluating the effects of glucose deprivation on acute hypoxic sensitivity

Augmentation of hypoxic sensitivity by mild mitochondrial inhibition using 3.3 mM NO_2^- has been demonstrated in Chapter 4. These data supported the hypothesis that hypoxic sensing in the CB is centred on a reduction in mitochondrial electron transport. Earlier in this chapter it was shown that glucose deprivation did not acutely stimulate the CB. However, it was put forward that a very small down-regulation in mitochondrial electron transport caused by glucose deprivation, although not great enough to elicit stimulation on its own, may sensitise the CB to acute hypoxia.

Paired hypoxic responses were recorded in either 11 mM glucose or glucose free solutions from the same chemoafferent fibre. The initial PO_2 was 300 mmHg and slowly decreased until the CB chemoafferent discharge frequency started to increase exponentially. At a frequency of approximately 10–12 Hz (below levels of maximal excitation), the response was rapidly terminated by switching to a 95% O_2, 5% CO_2 superfusate.

An example of the hypoxic response curves performed in 11 mM glucose or 0 mM glucose measured from a single fibre is shown in Figure 5.8A. Paired analysis showed that to attain a discharge frequency of 5 Hz, the PO_2 was lower in the glucose free superfusate, indicating a ‘left shift’ of the hypoxic response curve (Figure 5.8B). Calculating the difference in discharge frequency at selected PO_2 levels during hypoxia identified a significant reduction in frequency induced by glucose deprivation at a superfusate PO_2 of 125 mmHg (Figure 5.8C). Collectively, these data imply that glucose deprivation evoked a slight, but significant, attenuation of the CB response to hypoxia.

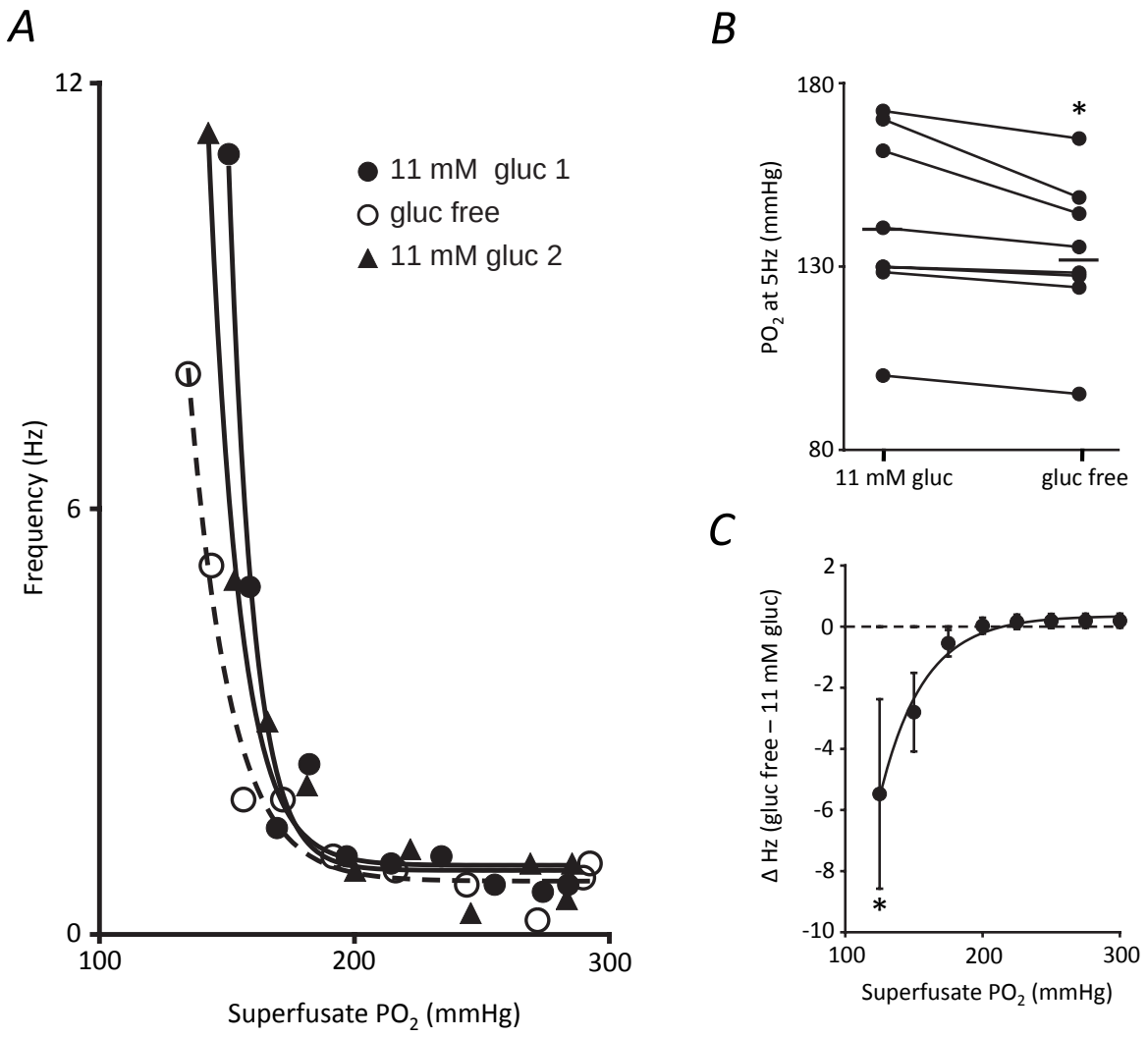


Figure 5.8 Glucose deprivation moderately attenuates the carotid body response to hypoxia. Data presented was taken from 8 chemoafferent fibres from 6 CB preparations. A) An example of the hypoxic response curves recorded from a single chemoafferent fibre from a single CB preparation observed in either glucose free or 11 mM glucose superfusates. B) Paired data showing the PO_2 required to attain a frequency of 5Hz in 11 mM glucose and glucose free respectively. * denotes $P < 0.05$ compared with PO_2 in 11 mM glucose; paired t-test. C) The calculated difference in discharge frequency in glucose free or 11 mM glucose measured at selected levels of superfusate PO_2 during hypoxia. Error bars indicate mean ± SEM. * denotes $p < 0.05$ compared with the difference at the PO_2 of 300 mmHg; one way repeated measures ANOVA with Dunnett's post-hoc comparisons test.

5.2.7 The impact of carotid body tissue incubation on the subsequent response to glucose deprivation

Published evidence has demonstrated that long term (> 24 hours) culture CB preparations had an acute sensitivity to physiological low glucose (246, 248). In these studies, CB tissue was cultured in hyperoxic conditions. Whether prolonged hyperoxic incubation of the intact CB directly sensitises it to low glucose was investigated in the experiments performed in this section.

Freshly isolated CB tissue was immediately placed in ice-cold bicarbonate buffered Krebs solution equilibrated with 95% O_2 and 5% CO_2 (severe hyperoxia) as described in Chapter 2. Tissue was incubated under these conditions for less than one hour (< 1 hr, hyperoxia), between 4 and 8 hours (4–8 hr, hyperoxia) or for 24 hours (24 hr, hyperoxia), before experiments proceeded. Upon recording, a baseline level of chemoafferent frequency was established between 0.25 and 1.25 Hz in 11 mM glucose by adjusting the superfusate PO_2, before exposing the CB to glucose deprivation. Characteristic examples of the responses to glucose deprivation following each incubation time period in hyperoxia are shown in Figure 5.9A. Incubation of tissue for 4–8 hours reduced the time taken to respond to glucose deprivation by approximately 30% compared with the < 1 hr, hyperoxia group (Figure 5.9B). A greater reduction in the time of approximately 65% was observed after 24 hours of incubation (Figure 5.9B).

Additional experiments were performed to examine if ROS production during the incubation period was necessary for modifying the CB response to glucose deprivation. Tissue was incubated in an ice-cold bicarbonate buffered Krebs solution for 24 hours equilibrated with 95% O_2, 5% CO_2 or air and 5% CO_2, in the presence of 1 mM Tempol

(superoxide scavenger). Treatment of the CB with Tempol for 24 hours under these conditions did not significantly restore the time taken to respond to subsequent glucose deprivation (Figure 5.9B). In all groups the initial basal discharge frequency and the peak responses to glucose deprivation were not significantly different. These data suggest that incubation of the CB in hyperoxia prior to experimentation does impair the ability of the CB to maintain discharge in the absence of superfusate glucose (thereby diminishing the time taken to respond) but this does not appear to be dependent on the generation of ROS.

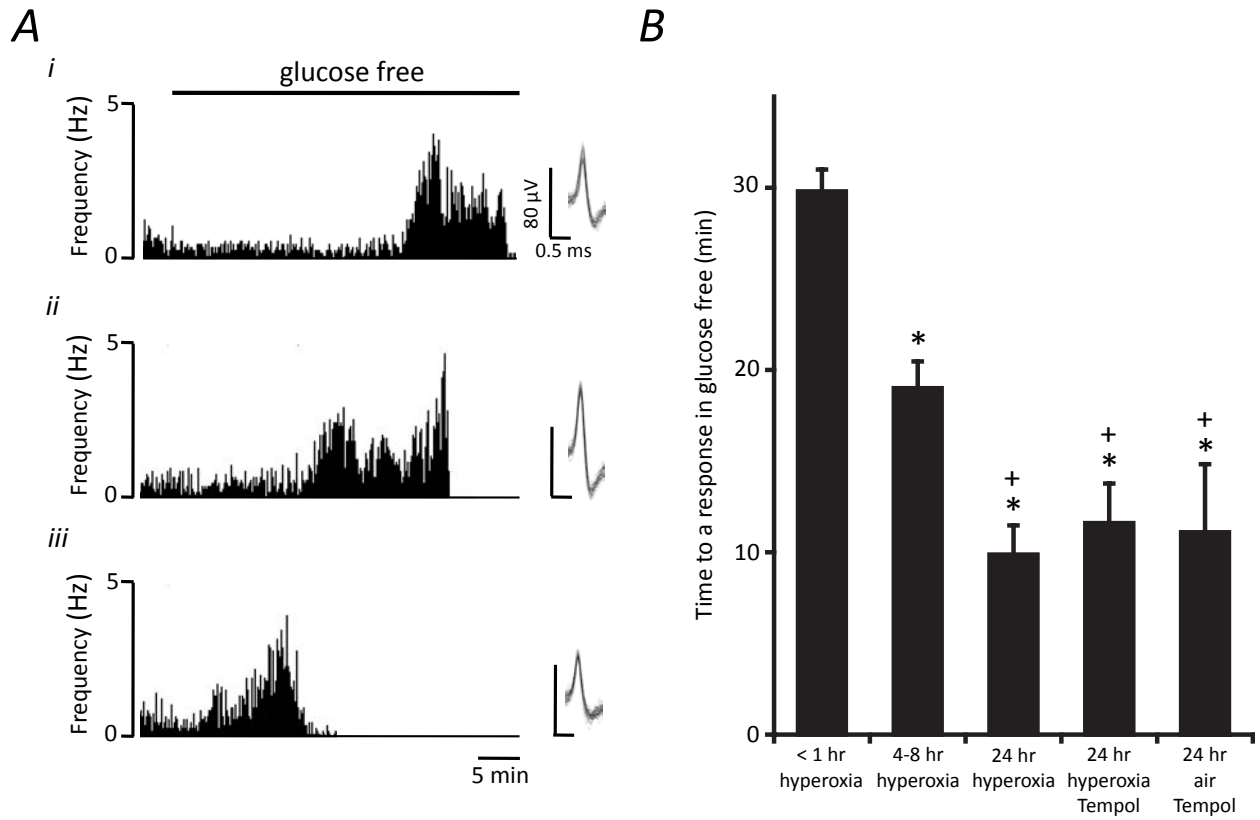


Figure 5.9 The duration of hyperoxic tissue incubation but not the generation of reactive oxygen species alters the time for the carotid body to respond to subsequent glucose deprivation. Example traces showing the response to glucose deprivation are demonstrated in CBs following incubation in ice-cold bicarbonate buffered Krebs equilibrated with 95% O_2, 5% CO_2 for less than 1 hour (*i),* between 4 and 8 hours (*ii*) and after 24 hours (*iii*). Chemoafferent single fibre discharge is expressed in frequency histograms binned in 10 second intervals. For each trace multiple action potentials have been overdrawn to show single fibre discrimination. B) Grouped data showing the impact of hyperoxic incubation, in the presence or absence of Tempol (ROS scavenger), on the subsequent time taken to respond to glucose deprivation. Data presented is taken from 10 fibres from 8 CB preparations (< 1 hr, hyperoxia), 8 fibres from 5 CB preparations (4–8 hr, hyperoxia), 8 fibres from 7 CB preparations (24 hr hyperoxia), 3 fibres from 3 CB preparations (24 hr, hyperoxia, Tempol) and 3 fibres from 3 CB preparations (24 hr, air, Tempol). Error bars indicate + SEM, * denotes a significance level of $P< 0.05$ compared with the time to a response in the < 1 hr, hyperoxia group, + denotes a significance level of $P< 0.05$ compared with the time to a response in the 4-8 hr, hyperoxia group; one way factorial ANOVA with Bonferroni post hoc analysis.

5.2.8 The impact of glucose deprivation on dissociated rat carotid body type I cells

Preliminary investigations monitoring changes in the type I cell $[Ca^{2+}]_i$ were performed to investigate the potential origin of the response to glucose deprivation. Dissociated type I cells were experimented on within 8 hours of CB isolation. The control superfusate was equilibrated at 10% O_2, to obtain a PO_2 of approximately 80 mmHg. This value is consistent with the PO_2 used in the studies performed on the CB slice and co-culture preparations that reported an acute sensitivity to low glucose (246, 248). $[Ca^{2+}]_i$ elevations in response to 0% O_2 were recorded initially to confirm normal type I cell function (Figure 5.10). Exposure of the type I cells to glucose deprivation for 40 minutes did not stimulate any increase in $[Ca^{2+}]_i$ above baseline levels (Figure 5.10). The magnitude of the hypoxia induced elevation in $[Ca^{2+}]_i$ during glucose free exposure was also suppressed compared to that measured in 11 mM glucose (Figure 5.10). Similar results were observed in one other type I cell cluster dissociated from a CB isolated from a different animal. These preliminary findings indicate that the chemoafferent response to glucose deprivation described earlier in this chapter may not have been of type I cell origin.

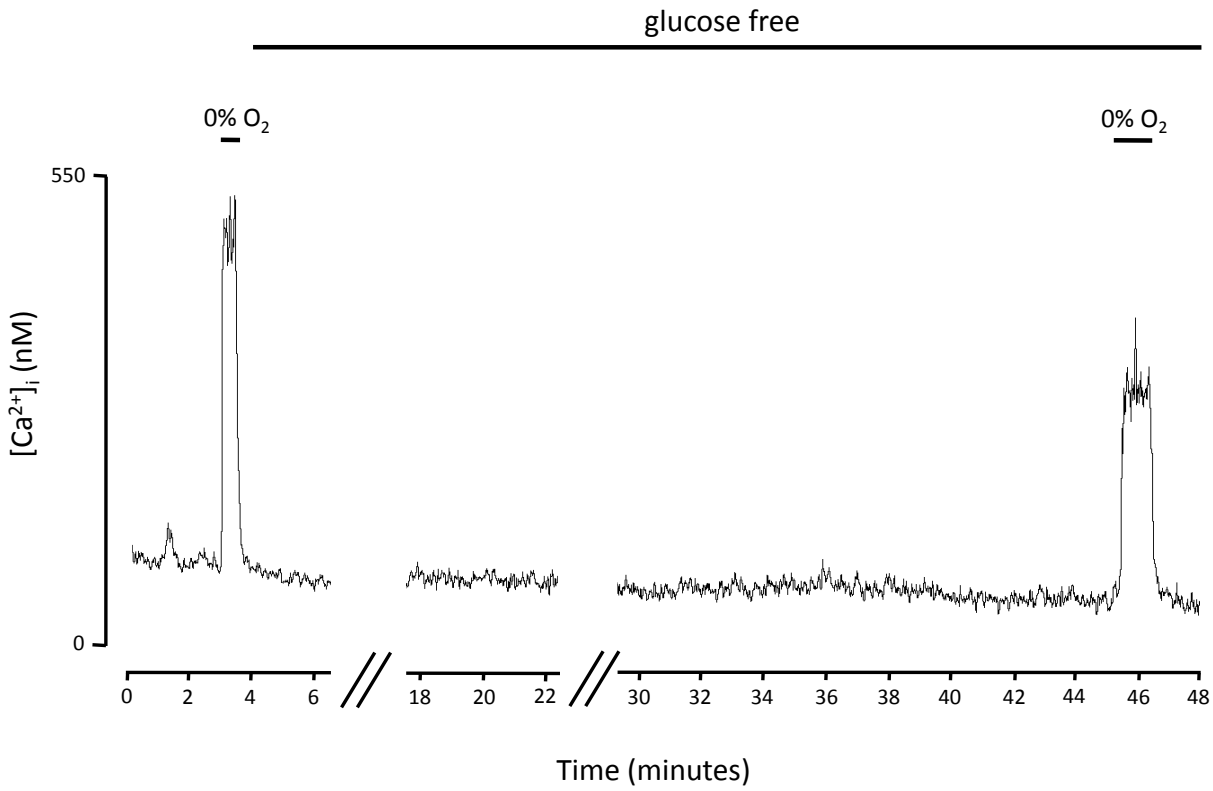


Figure 5.10 Dissociated type I cells do not show an increase in $[Ca^{2+}]_i$ in response to glucose deprivation for up to 40 minutes of exposure.
An example spectrum demonstrating the impact of hypoxia and glucose deprivation on $[Ca^{2+}]_i$ in type I cells. Responses to hypoxia were intact indicative of normal type I cell function. Glucose deprivation failed to enhance the type I cell $[Ca^{2+}]_i$ at any point over the course of the 40 minute period of exposure.

5.3 Chapter synopsis and discussion

5.3.1 Key Findings

The main points demonstrated in the current chapter are outlined below:

- The intact CB did not respond acutely to glucose deprivation at a normoxic basal discharge frequency of 0.25–1.5 Hz. A response was observed only after at least 20 minutes of sustained glucose free exposure.
- The time taken to respond to glucose deprivation was significantly reduced by raising basal chemoafferent activity or by prior exposure to severe hypoxia.
- Tyrosine hydroxylase positive type I cell clusters stained positively for both glycogen synthase I and glycogen phosphorylase BB.
- Pharmacological inhibition of glycogen phosphorylase decreased the response time to glucose deprivation.
- A response to glucose deprivation was immediately reversed by 1 mM glucose.
- The response to glucose deprivation was abolished or rapidly reversed by lactate and pyruvate.
- Glucose deprivation moderately attenuated the CB chemoafferent response to hypoxia.
- The time taken to respond to glucose deprivation was dependent on the duration of CB tissue incubation following isolation from the animal.
- Freshly isolated type I cells showed no sensitivity to glucose deprivation for up to 40 minutes of exposure.
- These data do not support the proposal that the CB is a physiological low glucose sensor.

5.3.2 The mechanism of glucose free induced chemoexcitation in the intact carotid body

Low glucose sensitive neurones in the brain are thought to be stimulated primarily as a consequence of a down-regulation in cellular energy status leading to direct activation of AMPK (235, 236). Some reports propose that a reduction in Na^+/K^+ pump activity subsequent to impaired glycolysis may also evoke GI neuronal excitation (237). Central to both of these mechanisms is a low glucose induced run-down in metabolism and ATP synthesis. Consistent with GI neurones, it has been suggested that CB stimulation evoked by glucose deprivation is a consequence of decreased ATP generation (249). However, evidence has also been described indicating that alterations in glucose metabolism and $[ATP]_i$ were not necessary for low glucose induced type I cell excitation, (246, 253). This implied that the CB contained some form of unidentified glucose sensitive receptor, maybe analogous to those present in the gastro-intestinal tract. Experiments performed in this study aimed to resolve these contradictory observations and conclusively support the hypothesis that chemoafferent stimulation following prolonged periods of glucose deprivation is purely dependent on the complete inhibition of glycolysis and a reduction in ATP production.

Pyruvate is the final product of glycolysis. Lactate can generate pyruvate and is utilised as an alternative metabolic substrate by some central neurones during glucose free exposure (357). In the current investigation, addition of lactate and pyruvate completely abolished the CB response to glucose deprivation. These substrates also immediately reversed the increase in chemoafferent activity caused by the absence of superfusate glucose. Thus, substrates capable of by-passing the glycolytic pathway and rapidly

entering the TCA cycle to drive ATP synthesis effectively nullified any glucose free induced stimulation.

In the vast majority of recognised mammalian glucose sensors, cell activation or deactivation is achieved through the direct modulation of glycolysis and $[ATP]_i$ (211, 227, 232). Central to the control of glycolysis in these cells is the highly specialised enzyme glucokinase (211, 227-229, 361). Glucokinase has a very high K_m for glucose (approximately 8 mM) compared with all other hexokinases that are in the µM range (215, 216). This ensures precise control of glycolysis over the full range of physiological glucose concentrations and makes these cells exquisitely sensitive to small fluctuations in plasma glucose (216).

In the CB, the absence of glucokinase in the type I cell (253) indicates that the rate of glycolysis will only begin to be impaired at very low concentrations of intracellular glucose. In the current study, the response to prolonged glucose deprivation was completely reversed by only 1 mM glucose. This strongly suggests that the stimulation was purely a consequence of time dependent run-down in glycolysis following the complete depletion of intracellular glucose. This could be the justification as to why a complete lack of chemoexcitation was observed in other intact CB preparations where 2 mM glucose was used as the low glucose stimulus (242, 243). Detection of a response only in the complete absence of glucose and not over a physiological range supports the proposal that the CB cannot function as an effective physiological glucose sensor.

5.3.3 The intact carotid body did not respond acutely to glucose deprivation; a role for glycogen

The central aim of the chapter was to characterise the response of the intact freshly isolated CB to prolonged glucose deprivation. The lack of an acute response to physiological low glucose has been described previously in this intact preparation (242, 247). Other different CB preparations appear to have a degree of intrinsic low glucose sensitivity (246, 248). It has been speculated that the apparent absence of low glucose sensitivity in the intact CB was due to the high level of PO_2 used in those investigations (248).

In experiments described in this thesis, the basal chemoafferent activity was adjusted to frequencies (0.25–1.5 Hz) consistent with those observed *in vivo* in arterial normoxia, in the rat (41). At this level of basal discharge frequency, exposure of the CB to glucose deprivation did not produce any further elevation in chemoafferent activity for at least 20 minutes. Increasing basal chemoafferent frequency to levels consistent with moderate hypoxia did reduce the time taken to respond. Even at these relatively high frequencies (6-8 Hz), the stimulation was still not rapid, taking at least 4 to 5 minutes to begin. In view of these findings it is suggested that the absence of low glucose sensitivity reported in previous studies was not a consequence of a high background PO_2. It could be argued that a PO_2 of 90 mmHg used on the monolayer of type I cells co-cultured with petrosal neurones (that showed intrinsic low glucose sensitivity) (248) is relatively hyperoxic compared to what these cells would be exposed to *in vivo*. Therefore, another mechanism independent of PO_2 must account for the low glucose sensitivity of these cultured type I cells.

Maintenance of basal chemoafferent discharge in glucose free conditions demonstrated that the CB tissue was able to sustain metabolic activity in the complete absence of extracellular substrate delivery. This is probably due to the presence of a significant level of intracellular metabolic reserve within either the type I cell, the afferent neurone or another neighbouring cell. In the complete absence of glucose uptake, glycogen metabolism would be fundamental for the preservation of glycolysis. In the brain tissue, astrocyte glycogen metabolism has been shown to increase during global ischaemia *in vivo* (362) and during glucose deprivation *in vitro* (355, 363). Functionally, regional escalations in astrocytic glycogenolysis have been shown to preserve central neuronal cell viability during glucose deprivation (364). It now appears that Schwann cell glycogen metabolism is essential in supporting the metabolism and function of peripheral myelinated neurones during prolonged glucose deprivation (358).

In this investigation, immunohistochemical analysis of CB sections positively identified both glycogen synthase I and glycogen phosphorylase BB in type I cell clusters. This implies that the type I cell is able to synthesise and metabolise glycogen. Pharmacological inhibition of glycogen phosphorylase significantly decreased the time taken for the CB to be stimulated by glucose deprivation. This outlined a functional role for glycogen in supporting chemoafferent function in the absence of external glucose consumption. However, the response was still not immediate, potentially signifying a residual degree of glycogen metabolism. A commercially available inhibitor selective for glycogen phosphorylase BB is not available and the DAB concentration used (100 µM) may have not been sufficient to completely ablate glycogenolysis in this intact CB preparation. The concentration of 100 µM DAB used was 5 fold higher than that

previously shown to partially impair hepatocyte glycogenolysis, in order to try and overcome diffusion limitations present in this whole organ preparation. A very recent study showed that glycogen metabolism in Schwann cells was dramatically impaired using 1 mM DAB (358). These cells were also incubated for 2 hours in the presence of DAB before experimentation. On reflection, a similar concentration and time of incubation in DAB may have further reduced the time taken for the CB to respond to glucose deprivation and this would have more conclusively established a functional role for glycogen in the CB.

It is established that hypoxia increases the rate of glucose uptake into the CB and this is highly indicative of an increase in glycolysis and glucose utilisation (352). The key allosteric activator of glycogen phosphorylase BB is AMP (365). Potential hypoxia evoked increases in AMP (see Chapter 4) would favour the metabolism of glycogen in the CB. Together these two mechanisms are predicted to have enhanced CB glycogen depletion in severe hypoxia. It is proposed that this run-down of glycogen accounted for the dramatic reduction in time taken for the CB to be stimulated by glucose deprivation following exposure to severe hypoxia. During glucose free exposure, a diminished initial store of glycogen would be consistent with a more rapid run-down in glycolysis and a faster reduction in the cellular energy status. Direct biochemical measurements of tissue CB glycogen content were not made because of the exceedingly small quantity of tissue. Cellular quantification of glycogen may be estimated by use of electron microscopy (358). Using this method, future experiments may focus on monitoring CB glycogen content to confirm the presence of glycogen in type I or other cells and depletion following severe hypoxia, ischaemia or glucose deprivation.

5.3.4 Is the chemoafferent response to glucose deprivation of type I cell origin?

The chemoafferent response to prolonged glucose deprivation could be a consequence of type I cell depolarisation, Ca^{2+} influx and neurosecretion leading to an increase in post-synaptic action potential generation. Alternatively, the increase in frequency could be the outcome of an increase in afferent neuronal excitability independent of any type I cell activation.

The chemoafferent response to prolonged glucose free exposure is observed approximately 20–40 minutes after the initial exposure. Preliminary data presented in this chapter indicates that dissociated type I cells are not stimulated by glucose deprivation for at least 40 minutes. The reason for this discrepancy was not definitively resolved. The chemoafferent responses to glucose deprivation and hypoxia are both suggested as being highly dependent on a reduction in cellular energy status. If this metabolic stress acted through the type I cell following prolonged removal of superfusate glucose (as it does in hypoxia) then the degree of chemoafferent excitation evoked by hypoxia and glucose deprivation may be expected to be equivalent.

The findings from this chapter identified that the magnitude of enhanced chemoafferent activity observed during prolonged glucose deprivation was only approximately 15% of that induced by hypoxia. It may be that the signalling within the type I cell following a reduction in energy status is fundamentally different between hypoxia and glucose deprivation. This is highly unlikely given that AMPK is activated by hypoxia and glucose deprivation (5, 102). It is more probable that the response to glucose deprivation is purely of neuronal identity and this would account for the discrepancy between cellular and whole organ responses described in this chapter.

Prolonged glucose deprivation does stimulate peripheral neurones. Consistent with data from the current study, a rise in the rate of spontaneous axonal depolarisations was reported in neurones in the optic nerve 35 to 40 minutes following removal of superfusate glucose (366). Electron microscopy has detected glycogen like granules in both the synaptic and non-synaptic elements of the sensory nerve endings in the CB (367, 368). The proposal that the lack of an acute response to glucose free media was dependent on glycogen remains plausible even if the excitation is purely of neuronal origin. The type I cell may contain more glycogen than the afferent neurone. On the other hand, a more rapid run-down of glycogen in the nerve ending compared with the type I cell, in normoxia or hypoxia, could be due to the increase in energy demand relied upon to generate neuronal action potentials. Calculations derived from neuronal activity estimated that an augmentation in single fibre frequency from 0 to 6 Hz increased ATP consumption by 8 fold (369). Following faster glycogen depletion in the afferent neurone, glycolysis would be impaired leading to a metabolic compromise before having any effect on the type I cell. Linking a depression in cellular energy status with an elevation in neuronal excitability could be through impairment of the Na^+/K^+ ATPase but a mechanism of this nature is still to be confirmed.

5.3.5 The effect of low glucose on the carotid body response to acute hypoxia

The mitochondrial hypothesis puts forward that chemoexcitation in hypoxia is dependent on a reduction in electron transport and the induction of metabolic stress. In the intact CB acute glucose deprivation was unable to increase chemoafferent frequency in basal conditions. Glucose deprivation has been shown to augment the CB response to acute hypoxia *in vitro* without stimulating the CB in more normoxic conditions (249, 251). In

this study, chemoexcitation evoked by hypoxia was slightly attenuated by glucose deprivation as evidenced by a small ‘left shift’ in the hypoxic response curve. The discharge frequency at any PO_2 was lower in the glucose free solutions and this became more exaggerated in more hypoxic conditions. $[Ca^{2+}]_i$ elevation in 0% O_2 was also inhibited in type I cells following 40 minutes of glucose deprivation.

A definitive explanation for this attenuation of the hypoxic response in glucose free conditions remains unidentified. Earlier findings in this thesis were consistent with the mitochondrial hypothesis and a hypoxia induced reduction in cellular energy status. However, the response to hypoxia may still require a significant amount of ATP consumption to drive many of the chemotransduction processes. An increase in ATP derived independently of the mitochondria may be attained by increased activation of adenylate kinase, or through an increase in the rate of glycolysis. The absence of extracellular glucose may have led to an inability to produce enough ATP through glycolysis to drive the full hypoxic response even when glycogen metabolism was maximal.

5.3.6 The effect of tissue incubation on the carotid body response to glucose deprivation

The data from the current study is consistent with those made in other studies indicating an absence of acute low glucose sensitivity in freshly isolated CB tissue (242, 243, 247, 249). Other studies using more long-term CB preparations including CB thin slices and co-cultures of type I cell clusters with petrosal neurones have reported rapid CB stimulation in response to low glucose (246, 248). Evidence from a recent investigation

showed that a single day of culture induced a degree of low glucose sensitivity in type I cells that was absent immediately following isolation (249).

The CB tissue that showed acute low glucose sensitivity appeared to have been cultured for a period of days under hyperoxic conditions. *In vitro* preparations incubated in hyperoxia are characterised by an increase in ROS production (370-372), an up regulation of anti-oxidant enzymes (373), DNA damage (374) and alterations in metabolism. More specifically, it has been reported that mitochondrial energy production is impaired by selective inhibition of succinate dehydrogenase and aconitase in the TCA cycle and ETC. It has been observed that hyperoxia induces a shift away from mitochondrial ATP generation in favour of anaerobic glycolysis (375). Pulmonary tissue exposed to hyperoxia for 7 days showed an increase in GLUT-1 and GLUT-4 mRNA and an increase in mRNA and protein expression of hexokinase II; both consistent with enhanced glucose utilisation (376). The same group also showed that ATP generated specifically through glycolysis was critical in maintaining lung tissue viability and preventing cell death in hyperoxia. Thus, it would seem that *in vitro* preparations exposed to prolonged periods of hyperoxia become more reliant on the availability of extracellular glucose uptake.

In this series of experiments, incubation of the whole CB organ in hyperoxia significantly decreased the time taken for it to be stimulated by glucose deprivation. The response time to glucose deprivation following 24 hours of hyperoxic incubation was only about 30% of that compared with freshly isolated tissue. These findings suggest that prolonged tissue incubation following isolation had a striking effect on the ability of the CB to maintain metabolism in the absence of superfusate glucose. Two hypotheses for

this are proposed. Firstly, incubation may have caused either a reduction in or an impairment of the capability of the organ to utilise metabolic glycogen reserves. Secondly, diminished TCA and ETC activity may have made the CB more dependent on glycolysis that generates far less ATP per molecule of glucose than total oxidative metabolism. 24 hour incubation either in lower PO_2 containing conditions or in the presence of Tempol (free radical scavenger) did not reverse the time of response of the CB to glucose deprivation. It is concluded that, in this instance, any variation in metabolic status seems to be independent of ROS generation.

The discrepancies in CB sensitivity to low glucose reported across a number of different investigations may be a consequence of two factors. Firstly, both groups that observed an acute sensitivity to low glucose used a method of decapitation before removing the CB tissue up to 5 minutes later. It is probable that this method caused a significant period of sustained ischaemia and severe hypoxia that increased CB metabolism and caused a dramatic reduction in metabolic glycogen reserves. Secondly, acute low glucose sensitivity was shown in tissue following a prolonged period (days) of incubation in conditions different to those *in vivo*. The acute low glucose sensitivity of this CB tissue is suggested as being a consequence of change in metabolic status of the cells following this incubation period leading to the total reliance on the extracellular glucose uptake for maintenance of glycolysis.

5.3.7 A role for the carotid body in glucose homeostasis

The findings described in this thesis are conceivably contradictory to previous reports, conclusively demonstrating that the CB is stimulated in hypoglycaemia *in vivo*. A number of studies have shown that CB activation has an important role in restoring plasma

glucose concentrations in response to hypoglycaemia, both in animals (240) and humans (241, 377). CB excitation in hypoglycaemia also appears to be fundamental in matching ventilation with the increase in metabolic rate and thereby preventing systemic acidosis (242, 243).

The global counter-regulatory response to hypoglycaemia is multi-faceted and highly integrated. Direct or indirect actions of hypoglycaemia include sympathetic activation (378), systemic hypokalaemia (379), cerebral vasodilatation (380), augmented ventilation and an increase in whole body metabolism (242). In addition, a number of counter-regulatory endocrine or neuro-endocrine factors are released into the systemic circulation including adrenaline, noradrenaline, cortisol and glucagon (245, 381). Consistent with the results identified in this thesis it is hypothesised that one of these factors released as a consequence of hypoglycaemia may stimulate the CB rather than low glucose *per se*. Of these the most probable CB activator may be a catecholamine given that a number of investigations have demonstrated that exogenous adrenaline and noradrenaline administration both elevated CSN activity and increased ventilation *in vivo* (382-384). This excitatory impact does however seem to show some inter-species variability, with intra-carotid administration of noradrenaline appearing to decrease ventilation in goats (385). Future experiments may aim to fully characterise the importance of adrenaline or noradrenaline in activating the CB and augmenting ventilation in hypoglycaemia. If catecholamines are found to stimulate the CB in humans then they may have an additional, and as yet unidentified, physiological role in enhancing ventilation during exercise.

5.3.8 Conclusions

The freshly isolated intact CB does not have an inherent sensitivity to physiological concentrations of low glucose and these observations indicate that it cannot act as a functional low glucose receptor in the whole animal. The maintenance of normal basal chemoafferent activity during glucose deprivation is supported by metabolism of glycogen to generate glucose that drives glycolysis. Prolonged glucose deprivation does evoke chemoafferent stimulation in the intact preparation and this is probably a consequence of a time dependent depletion in cellular energy status in the afferent neurone and not the type I cell. The intrinsic low glucose sensitivity described in other CB preparations is proposed as being dependent on an alteration of metabolic status subsequent to tissue ischaemia during surgery or following long term incubation *ex vivo*. These findings do not rule out a role for the CB in glucose homeostasis in the whole animal but suggest that the CB is activated indirectly by another blood-borne stimulus released as a consequence of the systemic hypoglycaemia and not directly by low glucose.

6. A functional role for adenosine derived from ecto-5'-nucleotidase in mediating the carotid body responses to hypoxia, hypercapnia and mitochondrial inhibition.

6.1 Chapter introduction and overview

The previous three chapters have focused on the impact of changes in cellular energy status on initiating CB chemoafferent stimulation, either in hypoxia or prolonged glucose deprivation. The results presented in Chapters 3 and 4 indicate that ATP, ADP and AMP are important intracellular signalling molecules and determine the extent of type I cell stimulation by modifying AMPK activity in the presence of Lkb1. The final results chapter now switches to investigate the effect of extracellular ATP metabolism on CB function. ATP is recognised as an important excitatory neurotransmitter. Upon hypoxic or hypercapnic stimulation it is released from type I cells and acts on the post-synaptic membrane to induce action potential generation. ATP is highly unstable and in the presence of ectonucleoside triphosphate diphosphohydrolyase 1 (CD39) and ecto-5'-nucleotidase (CD73) it can be rapidly metabolised to form adenosine. Adenosine is a well established neuromodulator in the CNS but its effects in the CB are less well characterised. This chapter focuses on the potential of this 'pool' of extracellular adenosine, derived from ATP metabolism, to modulate CB excitability.

Previous studies have established that exogenous adenosine administration acutely increases the CSN firing frequency *in vivo* and *in vitro* (280-283). This stimulation is

coupled to an increase in minute ventilation (284, 285) and in humans augmentation of adenosine signalling has been shown to amplify sympathetic outflow in skeletal muscle subsequent to the increase in respiration (288). In hypoxia, pharmacological inhibition of adenosine receptors causes an attenuation of both the increase in CSN discharge frequency (291) and the acute phase of the ventilatory response *in vivo* (292). On isolated CB tissue, non-selective A_2 receptor antagonism by caffeine inhibits both the ^{3}H-CA release and CSN activity under basal conditions and during hypoxic stimulation (297). It has been proposed that the importance of adenosine on modulating chemoafferent activity is most obvious in conditions of mild hypoxia (10).

The identification of significant levels of extracellular adenosine within the CB has been reported in normoxic/hyperoxic conditions and the degree of endogenous production is now known to increase in hypoxia (11). A recent study demonstrated that the increase in adenosine production under mild hypoxic conditions was not further amplified in extreme hypoxia (10).

Adenosine may also be generated within the actual type I cell through intracellular ATP catabolism. It may be released through the bidirectional nucleotide transporter ENT thereby contributing to the overall synaptic adenosine concentration (290). Pharmacological inhibition of ENT appears not to modify CB extracellular adenosine concentrations in normoxia, but does attenuate the increase in synaptic adenosine during hypoxia (11). The same study reported that CD73 deactivation with AOPCP significantly reduced adenosine concentrations in both normoxia and hypoxia. As yet the relative functional significance of intracellular and extracellular derived adenosine on CB sensory activity has not been defined in normoxia, hypoxia, hypercapnia or in response

to mild mitochondrial inhibition associated with sLTF in animals pre-conditioned with CIH (313). With this in mind the experiments in the current chapter were designed to examine the functional impact of adenosine derived specifically from CD73 on the CB basal discharge frequency and on the chemoafferent response to hypoxia. A role for adenosine produced purely from CD73 activity in establishing the stimulus threshold required for chemoafferent activation in response to mild mitochondrial inhibition and hypercapnia was also investigated in later sections.

The aims of the studies in this chapter are summarised below:

1. To establish if adenosine derived from CD73 was important in the generation of the basal chemoafferent discharge frequency in normoxia/hyperoxia.

2. To examine if CD73 activity was significant in eliciting peak chemoafferent responses to severe hypoxia or in modifying the sensitivity of the CB to graded hypoxia.

3. To investigate if chemoafferent stimulation evoked by mild mitochondrial inhibition with NO_2^- was altered by impaired adenosinergic signalling or reduced CD73 activity.

4. To study if the functional CB response to hypercapnia had any dependence on adenosine derived specifically from CD73 or on cAMP generated from transmembrane adenylate cyclase.

6.2 Results

6.2.1 Neuromodulation of the carotid body response to hypoxia by adenosine generated from ecto-5'-nucleotidase

The first set of experiments in this chapter were designed to evaluate the functional significance of the CD73 derived adenosine on single fibre CSN activity under basal normoxic/hyperoxic conditions and in hypoxia.

Isolated intact CBs were superfused at a control PO_2 of 300 mmHg to establish an initial baseline firing frequency (Figure 6.1A). Although seemingly hyperoxic, it was hypothesised that due to the diffusion gradient across the tissue the PO_2 at the site of individual type I cell clusters would be significantly lower. The mean basal discharge frequency in 300 mmHg PO_2 was 0.58 ± 0.34 Hz (Figure 6.1B) and this value was consistent with *in vivo* recordings made in the rat in arterial normoxia. The superfusate PO_2 was then changed to a single fixed value of 60 mmHg (estimated as being severely hypoxic in the superfused intact CB preparation) to induce a maximal level of chemoafferent hypoxic discharge frequency. Paired experiments were performed in the presence and absence of 100 μM α,β-methylene ADP (AOPCP), an inhibitor of CD73. This concentration was consistent with that previously used on the whole intact CB preparation and is known to decrease adenosine production in both normoxic and hypoxic conditions (11).

The impact of AOPCP under normoxic/hyperoxic (superfusate PO_2 of approximately 300 mmHg) conditions was striking and almost completely abolished the basal chemoafferent activity (Figure 6.1A and B). However, this level of inhibition was not

maintained in severe hypoxia as evidenced by the increase in discharge frequency evoked at a superfusate PO_2 of 60 mmHg being equivalent in the presence and absence of AOPCP (Figure 6.1C).

Further experiments examined a potential effect of CD73 generated adenosine on the chemoafferent response to graded hypoxia. Rather than switching to a single level of severe hypoxia, the superfusate PO_2 was slowly reduced from 300 mmHg to approximately 100 mmHg before being rapidly reversed to avoid potential organ damage or neurotransmitter depletion. A characteristic example of the discharge frequency recorded from a single fibre during graded hypoxia in the presence and absence of AOPCP is shown in Figure 6.2A. The individual hypoxic response curves obtained from the same fibre are plotted in Figure 6.2B, showing a characteristic reduction in the PO_2 threshold required for initiation of hypoxia response coupling evoked by concomitant application of AOPCP. The inhibition caused by AOPCP was rapidly reversible and the original response to hypoxia was almost fully recovered following removal of the agent from the superfusate (Figure 6.2A and B).

In order to quantify this change in PO_2 threshold or ‘left shift’, PO_2 values were calculated from the response curves when the discharge frequency was at 5 Hz, and compared in the presence and absence of AOPCP. This frequency was selected as it lies on the exponential region of the curve but is not at a point at which the response may have begun to decline (see Chapter 2). Grouped paired measurements showed that AOPCP significantly reduced the superfusate PO_2 required to elicit a discharge frequency of 5 Hz by a mean value of approximately 30 mmHg and this reduction was consistent across all fibres (Figure 6.2C). This emphasised a significant role for

extracellular adenosine generated from CD73 in establishing the set point for the initiation of CB response to hypoxia.

Detailed analysis of individual hypoxic response curves showed that the exponential rate constant was significantly elevated in the presence of AOPCP (0.054 ± 0.009) compared with the paired controls (0.038 ± 0.006, n=8; paired t-test). This demonstrates that the rate of increase in discharge frequency upon onset of the response was greater in the presence of AOPCP. The shape of the response curve (an example of which is shown in Figure 6.2B) suggests that AOPCP effectively abolished the graded nature of chemoafferent response to hypoxia.

Data from individual response curves was used to calculate the difference in absolute discharge frequency in the presence of CD73 inhibition over a range of PO_2 values during hypoxia (Figure 6.2D). At all superfusate PO_2 levels between 300 and 100 mmHg, AOPCP depressed the single fibre discharge frequency. The magnitude of attenuation was significantly enhanced at PO_2 values of 125 and 100 mmHg (Figure 6.2D). Therefore, the absolute effect of CD73 inhibition on the chemoafferent activity exhibited a clear PO_2 dependence and this demonstrates that adenosine is significant in establishing the acute hypoxic sensitivity of the *in vitro* CB.

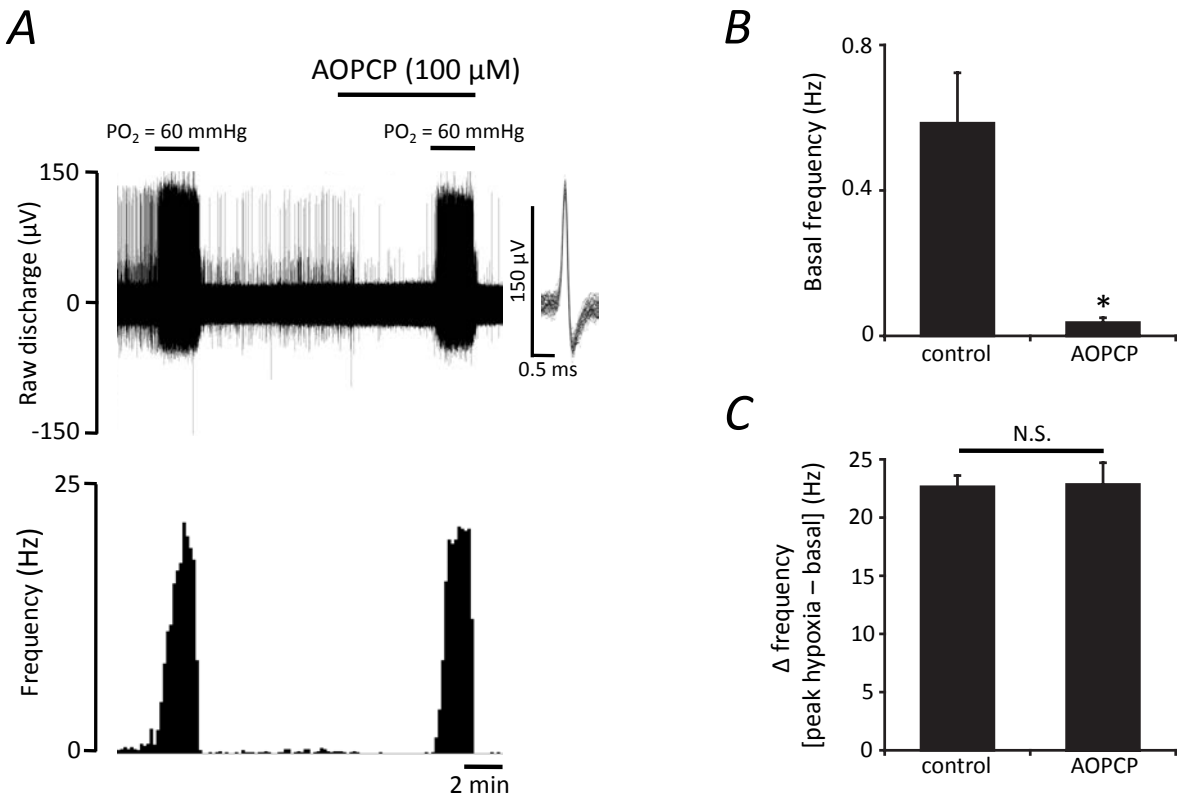


Figure 6.1 Inhibition of CD73 almost completely abolishes the chemoafferent basal discharge frequency but does not change the peak response to a single level of severe hypoxia.
A) An example trace of the effect of CD73 inhibition with 100 µM AOPCP on basal frequency and on the peak response to a single level of severe hypoxia. Raw discharge is shown (upper) along with frequency histograms (lower) that collate single fibre action potentials in 10 s intervals. Overdrawn action potentials are shown inset to demonstrate the single fibre discrimination from which the frequency was taken.
B) Mean basal frequency in the presence and absence of the CD73 inhibitor AOPCP. C) The mean change in frequency evoked by severe hypoxia (PO_2 ~ 60 mmHg) in the presence and absence of the CD73 inhibitor AOPCP. The data presented is taken from 6 fibres from 4 CB preparations. Error bars indicate + S.E.M. * denotes $P < 0.05$ compared with control frequency; paired t-test.

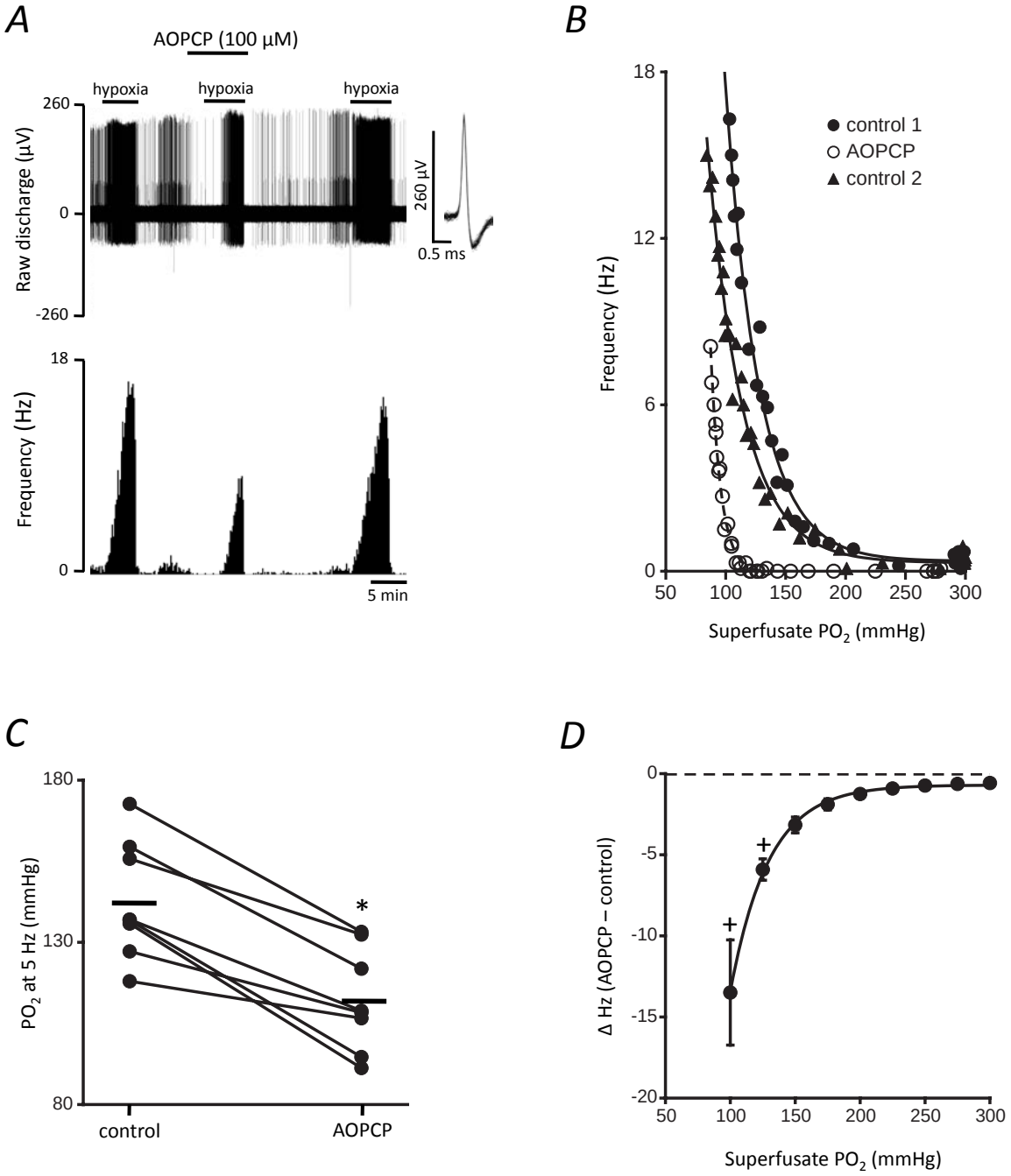


Figure 6.2 Adenosine generated from CD73 is important in establishing the hypoxic sensitivity of the *in vitro* carotid body.
A) Characteristic example of the response to graded hypoxia (300–100 mmHg PO_2) in the presence and absence of CD73 inhibition with AOPCP. Raw discharge is shown (upper) along with frequency histograms (lower) that collate single fibre action potentials in 10 s periods. Overdrawn action potentials are shown inset to demonstrate the single fibre discrimination from which the frequency was taken.
B) Individual hypoxic response curves taken from the same fibre recorded in A), showing a reversible 'left shift' in the presence of AOPCP. C) PO_2 values for each fibre when the discharge frequency was at 5 Hz in the first control and following addition of AOPCP. D) Calculated mean difference in discharge frequency evoked by AOPCP over a range of superfusate PO_2 levels. The data presented is from 8 fibres from 5 CB preparations. Error bars indicate ± S.E.M. For C), * denotes $P < 0.05$ compared with control PO_2; paired t-test. For D), + denotes $P < 0.05$ compared with the frequency difference at 300 mmHg PO_2; one way repeated measures ANOVA with Bonferroni post hoc analysis.

6.2.2 Neuromodulation of the carotid body response to nitrite by extracellular adenosine

Results from Section 6.2.1 demonstrated that adenosine derived from CD73 almost completely abolished basal chemoafferent discharge and altered the PO_2 threshold required for initiation of the response to hypoxia. These data support the idea that downstream effectors of adenosinergic signalling pathways are significant in establishing the hypoxic sensitivity of the CB. It was hypothesised that adenosine generated from CD73 may have a similar neuromodulatory role in regulating the CB sensitivity to other related or unrelated stimuli. As yet, the impact of adenosinergic signalling on the CB response to mitochondrial inhibition has not been investigated. Detailed examination of neuromodulators and their effects on CB responses to mild mitochondrial inhibitors may be of particular importance given that the sLTF associated following CIH is closely associated with mild inhibition of mitochondrial electron transport (313).

In these experiments, the rapidly reversible mitochondrial inhibitor nitrite (NO_2^-) was used to induce moderate elevations in chemoafferent discharge frequency consistent with those frequencies observed in sLTF following CIH (313). The CB was exposed to 10 mM NO_2^- for 5 minutes and a mean discharge frequency was taken from the final minute, once a relatively stable level of excitation had been achieved (Figure 6.3A and B). The non-specific adenosine receptor antagonist 8-SPT (300 μM) significantly diminished basal chemoafferent activity and attenuated the frequency attained upon stimulation with NO_2^- (Figure 6.3A and B). These inhibitory actions of 8-SPT were reversible as shown in the example trace in Figure 6.3A. The absolute increase in discharge frequency elicited by NO_2^- (NO_2^- – basal) in the presence of 8-SPT was

reduced in each fibre studied and the calculated mean NO_2^--induced frequency elevation with concurrent 8-SPT application was approximately 25% of that observed in control conditions (Figure 6.3C).

Inhibition of CD73 with 100 µM AOPCP produced even more striking results. An example trace demonstrating the impact of AOPCP on the response to 10 mM NO_2^- is shown in Figure 6.4A. AOPCP almost completely abolished basal discharge and eradicated the response to NO_2^- (Figure 6.4B). In every fibre tested, the absolute elevation in discharge frequency evoked by NO_2^- (NO_2^- – basal) was almost completely ablated in the presence of AOPCP (Figure 6.4C). These data indicate that chemoafferent stimulation evoked by mild mitochondrial inhibition with 10 mM NO_2^- is critically dependent on receptor mediated actions of adenosine generated from CD73.

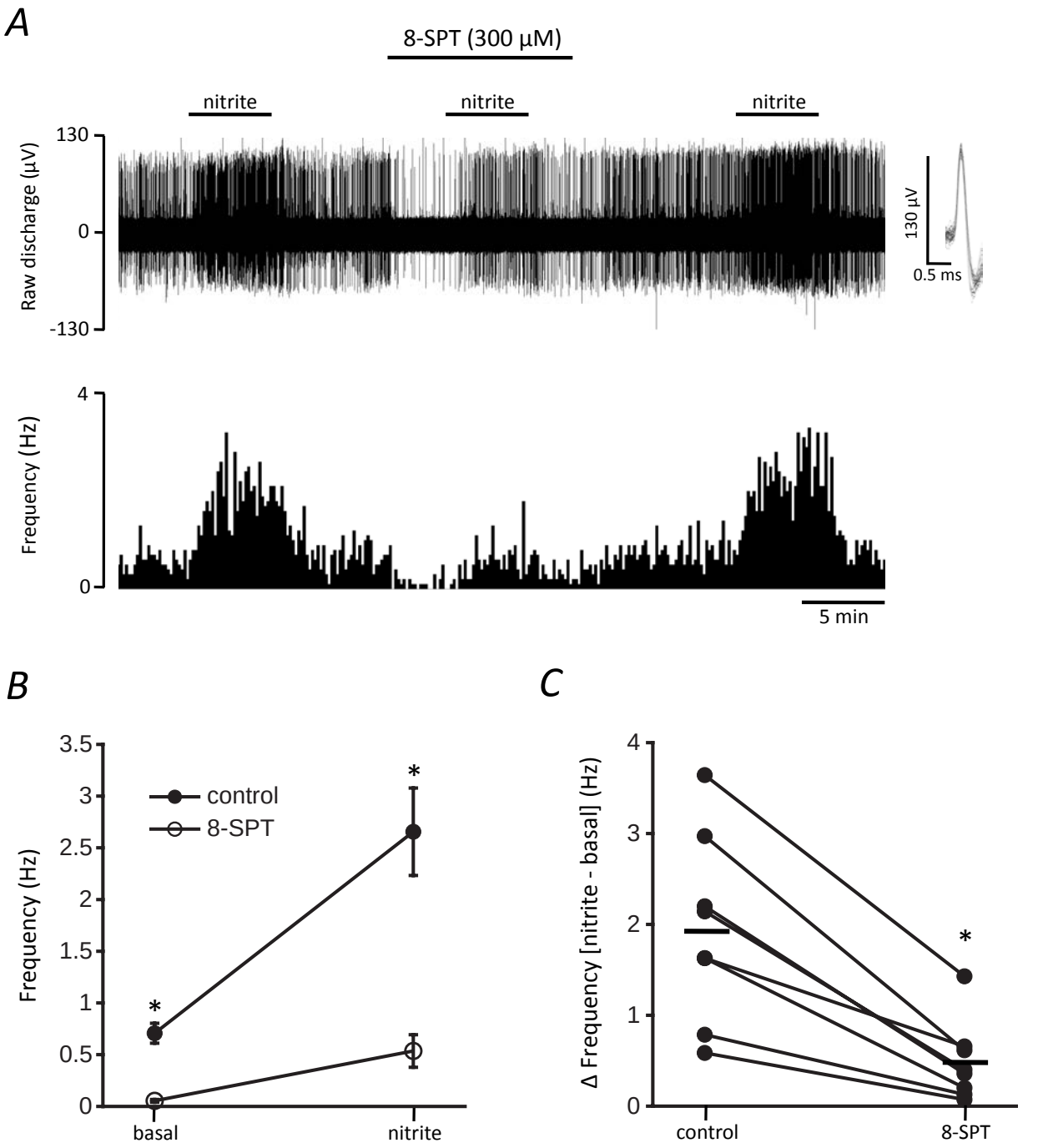


Figure 6.3 Adenosine receptor blockade attenuates the carotid body response to mitochondrial inhibition with 10 mM nitrite

A) Characteristic example recording of the response to nitrite (NO_2^-) in the presence and absence of the non-specific adenosine receptor antagonist 8-SPT. Raw discharge is shown (upper) along with frequency histograms (lower) that group single fibre action potentials in 10 s intervals. Overdrawn action potentials are shown inset to demonstrate the single fibre discrimination used to measure frequency. B) Mean discharge frequencies recorded under basal conditions and following addition of NO_2^-, in control and following addition of 8-SPT. The data presented is from 8 fibres from 5 CB preparations. Error bars indicate ± S.E.M. * denotes $P < 0.05$ compared with control frequency; one way repeated measures ANOVA with Bonferroni post hoc analysis test. C) Discharge frequency differences (NO_2^- – basal) for each fibre in the presence and absence of 8-SPT. * denotes $P < 0.05$ compared with control frequency; paired t-test.

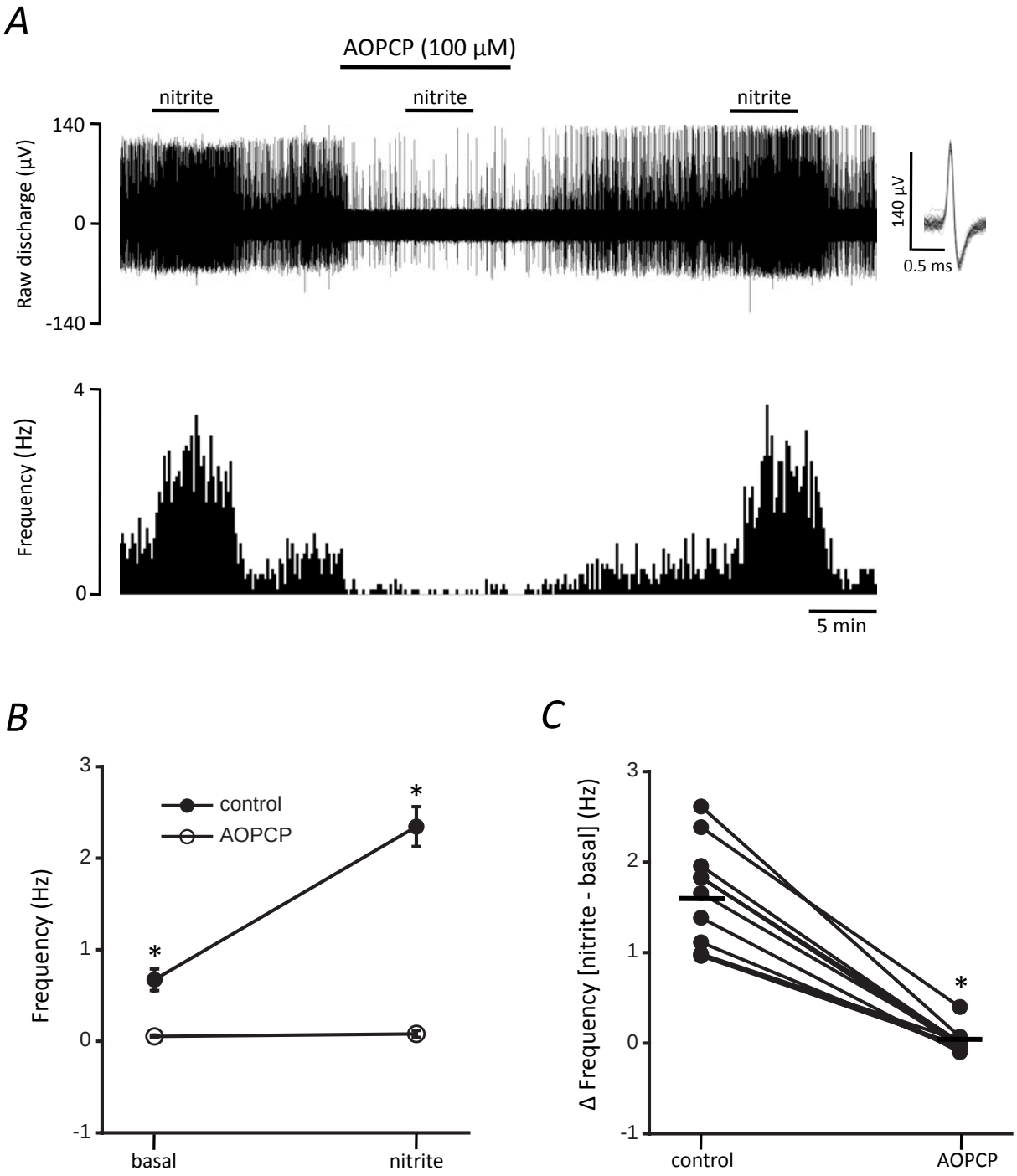


Figure 6.4 Adenosine derived from CD73 is a critical mediator of the carotid body response to mitochondrial inhibition with 10 mM nitrite.
A) Characteristic example recording of the response to nitrite (NO_2^-) in the presence and absence of the CD73 inhibitor AOPCP. Raw discharge is shown (upper) along with frequency histograms (lower) that group single fibre action potentials in 10 s periods. Overdrawn action potentials are shown inset to demonstrate the single fibre discrimination used to calculate the frequency. B) Mean discharge frequencies recorded under basal conditions and following addition of NO_2^-, in control and following addition of AOPCP. The data presented is from 10 fibres from 5 CB preparations. Error bars indicate ± S.E.M. * denotes $P < 0.05$ compared with control frequency; one way repeated measures ANOVA with Bonferroni post hoc analysis test. C) Discharge frequency differences (NO_2^- – basal) for each fibre in the presence and absence of AOPCP. * denotes $P < 0.05$ compared with control frequency; paired t-test.

6.2.3 Neuromodulation of the carotid body response to hypercapnia by extracellular adenosine

The importance of adenosine derived from CD73 on CB basal chemoafferent activity and responses to graded hypoxia or mild mitochondrial inhibition was observed in the previous two sections. These results are consistent with the idea that the neuromodulatory function of adenosine is to contribute to the resting excitability of the CB and to establish a threshold for activation in response to any stimulus. To investigate this idea further, measurements of chemoafferent activity were made in response to hypercapnia (another comparatively mild stimulus), in the presence and absence of pharmacological agents that impaired adenosinergic signalling pathways.

Experiments initially evaluated the effect of adenosine receptor antagonism on the chemoafferent response to hypercapnia. Addition of non-selective adenosine receptor antagonist 8-SPT (300 µM) to the superfusate significantly diminished discharge frequency in both normocapnic (40 mmHg PCO_2) and hypercapnic conditions (80 mmHg PCO_2) (Figure 6.5A and B). Measurements from all fibres showed that the absolute increase in discharge frequency evoked by hypercapnia was significantly depressed in the presence of 8-SPT (Figure 6.5C). The calculated CO_2 sensitivity (Δ Hz / mmHg PCO_2) following application of 8-SPT was approximately 20% of control (Figure 6.5D).

Selective inhibition of CD73 with 100 µM AOPCP almost completely abolished the single fibre discharge frequency recorded in both normocapnic and hypercapnic conditions (Figure 6.6A and B). This inhibitory effect was rapidly reversed once the drug was removed from the superfusate as demonstrated in the raw trace example in Figure 6.6A. Recordings from all single fibres showed that the absolute increase in discharge

frequency evoked by hypercapnia was decreased with AOPCP application compared with control (Figure 6.6C). The mean increase in discharge frequency in the presence of AOPCP upon hypercapnic stimulation was just above 0 Hz, demonstrating almost complete ablation of the response to hypercapnia (Figure 6.6C). Subsequent calculation of CO_2 sensitivity identified a remarkable reduction with AOPCP and again demonstrated that inhibitory targeting of adenosine derived from CD73 effectively abolished the functional response of the CB to hypercapnic stimulation (Figure 6.6D). In view of these findings, it is proposed that tonic generation of adenosine from CD73 has a functional role in establishing the CB sensitivity to hypercapnia.

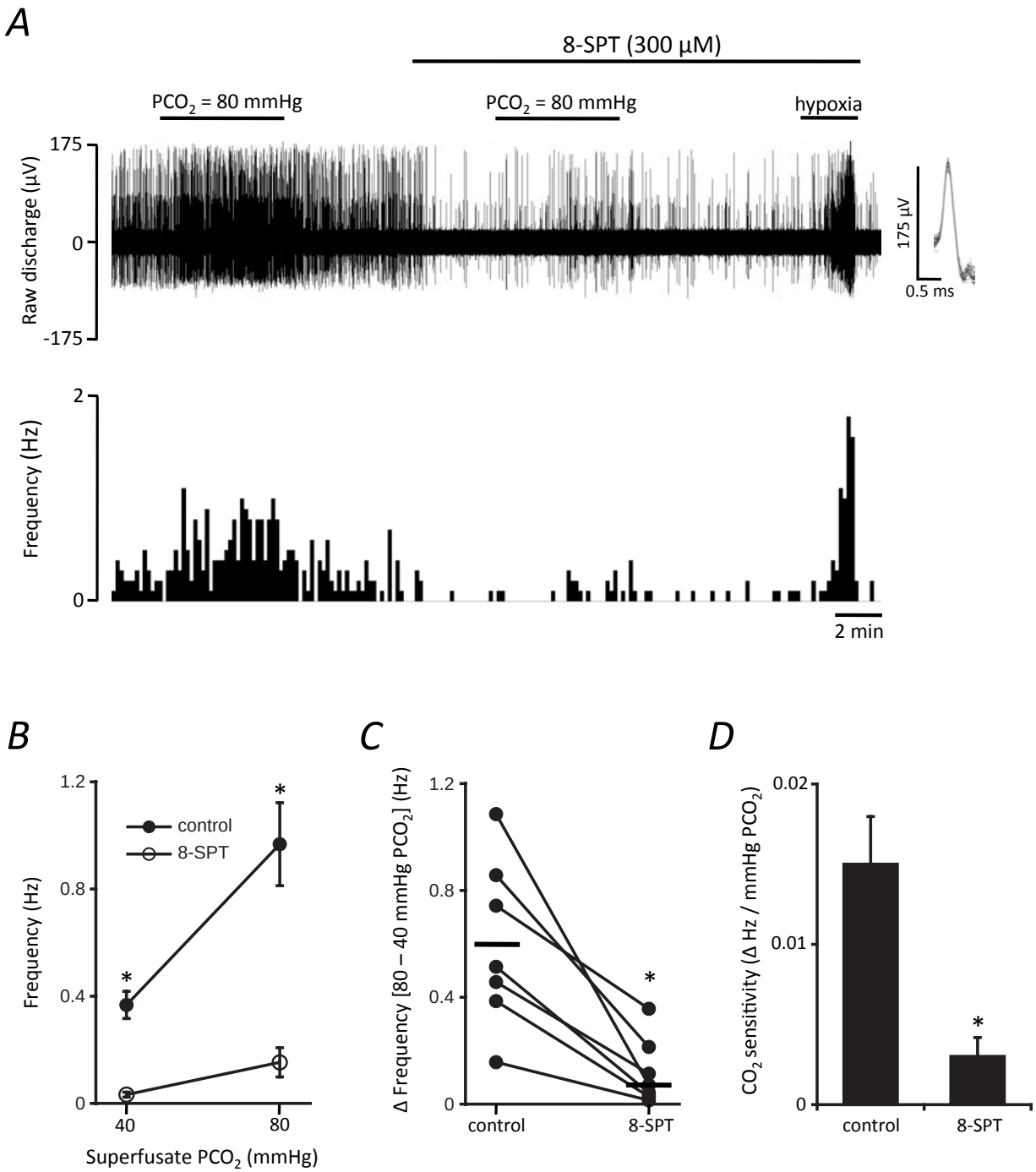


Figure 6.5 Adenosine receptor blockade attenuates the carotid body response to hypercapnia. A) Characteristic example recording of the response to hypercapnia in the presence and absence of the non-specific adenosine receptor antagonist 8-SPT. Raw discharge is shown (upper) along with frequency histograms (lower) that group single fibre action potentials in 10 s periods. Overdrawn action potentials are shown inset to demonstrate the single fibre discrimination used to measure frequency. B) Mean discharge frequencies recorded in normocapnia (PCO_2 = 40 mmHg) and hypercapnia (PCO_2 = 80 mmHg), in control and following addition of 8-SPT. C) Discharge frequency differences (80–40 mmHg PCO_2) for each single fibre in the presence and absence of 8-SPT. D) Calculated mean CO_2 sensitivity (Δ Hz / mmHg PCO_2) in control conditions and following 8-SPT application. The data presented is from 7 fibres from 3 CB preparations. Error bars indicate ± S.E.M. For B), * denotes $P < 0.05$ compared with control group; one way repeated measures ANOVA with Bonferroni post hoc analysis. For C) and D), * denotes $P < 0.05$ compared with control group; paired t-test.

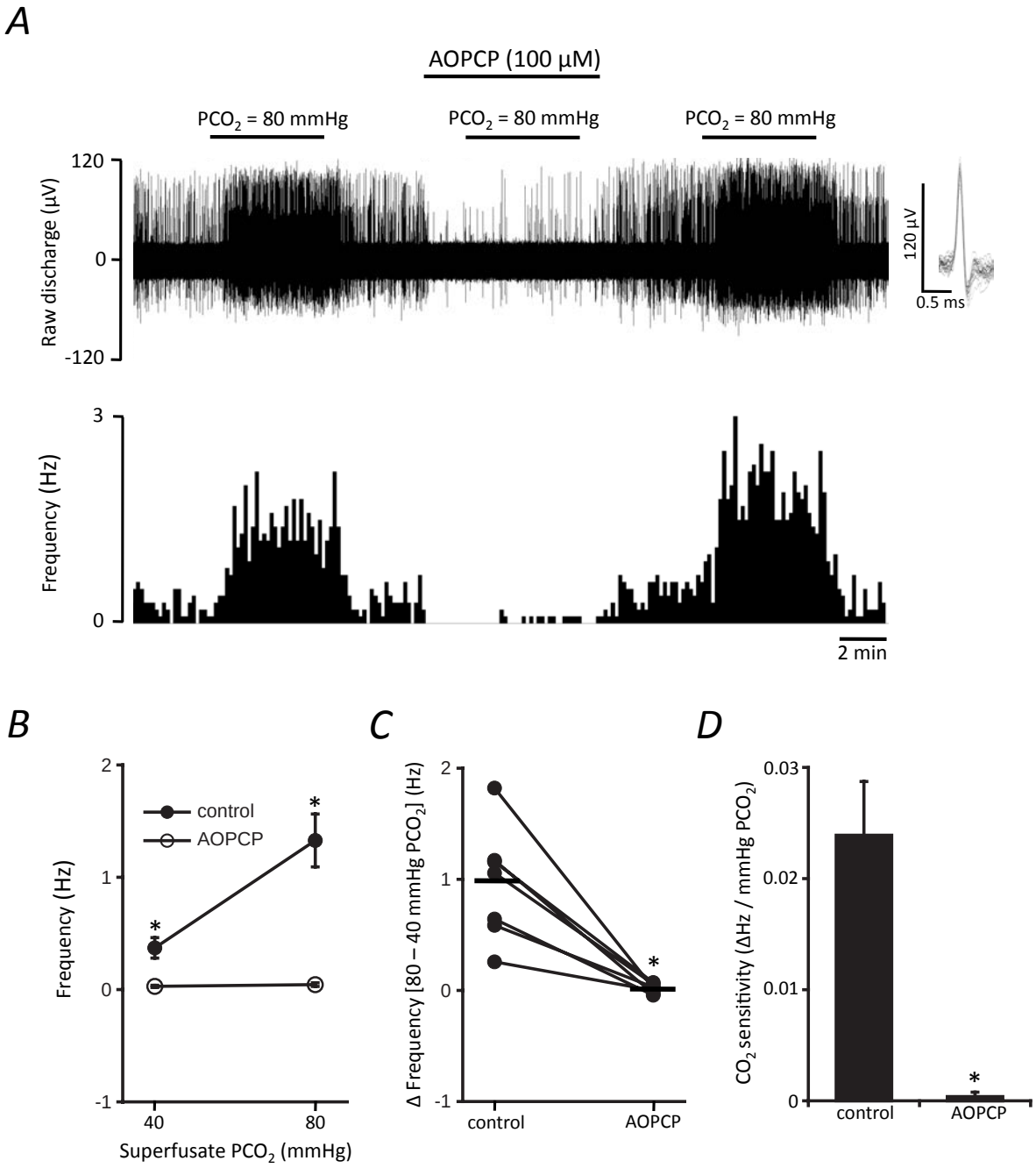


Figure 6.6 Adenosine derived from CD73 is a critical neuromodulator of the carotid body chemoafferent response to hypercapnia.
A) Characteristic example recording of the response to hypercapnia in the presence and absence of the CD73 inhibitor AOPCP. Raw discharge is shown (upper) along with frequency histograms (lower) that group single fibre action potentials in 10 s intervals. Overdrawn action potentials are shown inset to demonstrate the single fibre discrimination used to measure frequency. B) Mean discharge frequencies recorded in normocapnia (PCO_2 = 40 mmHg) and hypercapnia (PCO_2 = 80 mmHg), in control conditions and following addition of AOPCP. C) Discharge frequency differences (80–40 mmHg PCO_2) for each fibre in the presence and absence of AOPCP. D) Calculated mean CO_2 sensitivity (Δ Hz / mmHg PCO_2) in control conditions and following AOPCP drug application. The data presented is from 6 fibres from 5 CB preparations. Error bars indicate ± S.E.M. For B), * denotes $P < 0.05$ compared with control group; one way repeated measures ANOVA with Bonferroni post hoc analysis. For C) and D), * denotes $P < 0.05$ compared with control group; paired t-test.

6.2.4 The impact of cAMP generated from transmembrane adenylate cyclase on the carotid body response to hypercapnia

The final experiments were designed to investigate the potential role of effectors downstream of adenosine A_2 receptors in mediating CB chemoafferent excitability. Stimulation of G-protein coupled adenosine A_2 receptors increases neuronal excitability by directly activating transmembrane adenylate cyclases (tmAC) and augmenting $[cAMP]_i$. The significant inhibitory effects of CD73 deactivation on basal chemoafferent activity and on responses to a number of stimuli described earlier in the section were hypothesised as being primarily due to a decrease in basal $[cAMP]_i$ generation. Inhibition of tmAC has been shown previously to attenuate the response to hypoxia (386) and so was not studied in this section. However, the impact of direct inhibition of tmAC on the basal CB chemoafferent activity and on the functional sensory neuronal response to hypercapnia has not previously been examined.

Addition of the tmAC selective antagonist SQ22536 (200 μM) (387) elicited a rapid reduction of the basal chemoafferent discharge frequency in normocapnic conditions in all fibres tested (Figure 6.7A and B). In addition, the absolute mean discharge frequency observed upon hypercapnic stimulation was significantly attenuated by SQ22536 (Figure 6.7A and B). This inhibitory action of SQ22536 on normocapnic and hypercapnic discharge frequency was reversed upon removal of the agent from the superfusate as shown in the raw trace example in Figure 6.7A. In all fibres, the absolute increase in discharge frequency evoked by hypercapnia was depressed in the presence of SQ22536 (Figure 6.7C). The calculated CO_2 sensitivity was significantly reduced by SQ22536, measuring only approximately 60% of the control CO_2 sensitivity (Figure 6.7D). These data identify a functional role for cAMP derived from tmAC in setting the

basal chemoafferent discharge in normocapnia and in establishing a significant component of the CB sensitivity to hypercapnia.

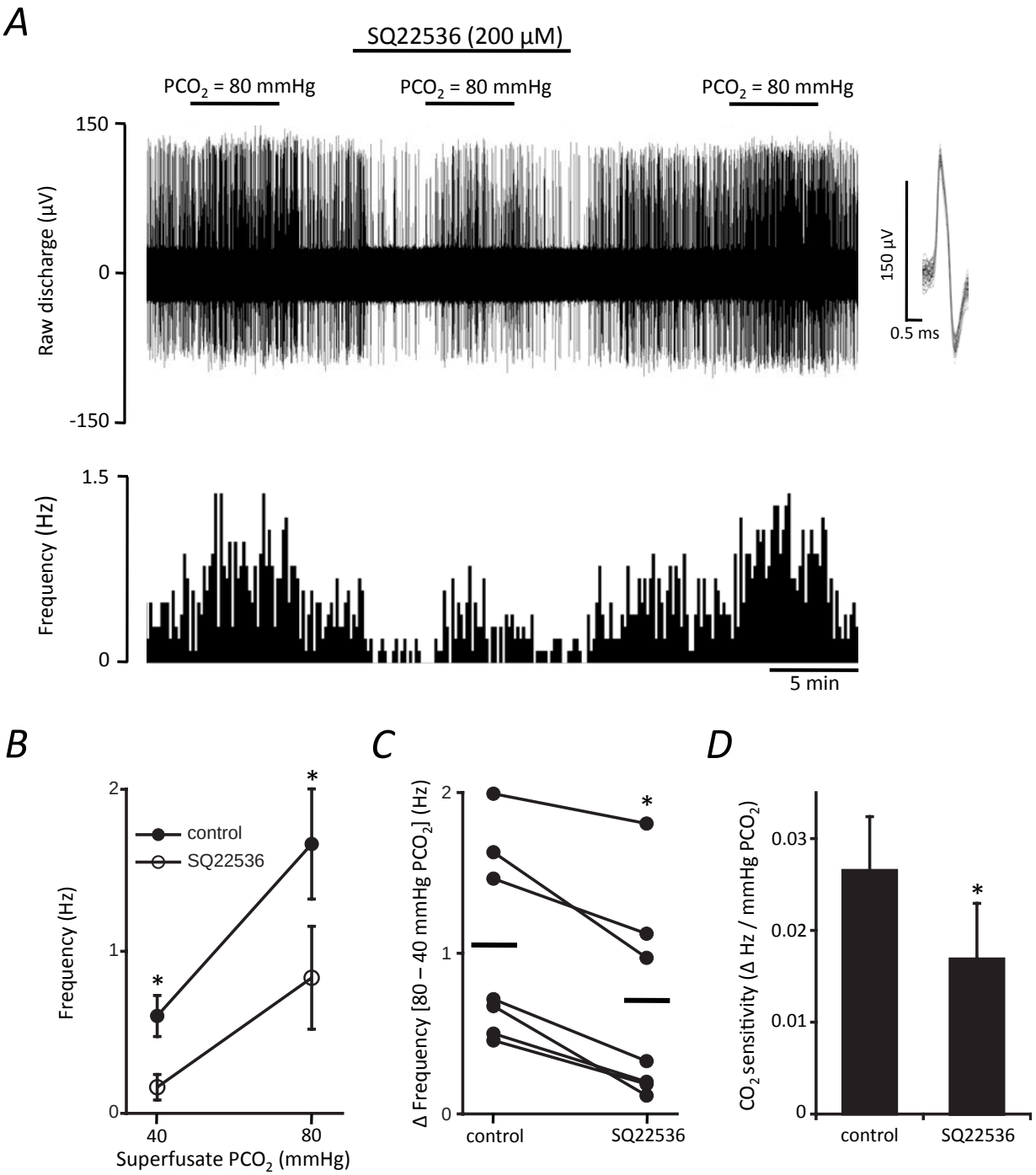


Figure 6.7 Pharmacological inhibition of transmembrane adenylate cyclase attenuates basal chemoafferent activity and the response to hypercapnia.
A) Characteristic example recording of the response to hypercapnia in the presence and absence of the tmAC inhibitor SQ22536. Raw discharge is shown (upper) along with frequency histograms (lower) that collate single fibre action potentials in 10 s periods. Overdrawn action potentials are shown inset to demonstrate the single fibre discrimination used to measure frequency. B) Mean discharge frequencies recorded under normocapnic (PCO_2 = 40 mmHg) and hypercapnic (PCO_2 = 80 mmHg), in control conditions and following addition of SQ22536. C) Discharge frequency differences (80–40 mmHg PCO_2) for each fibre in the presence and absence of SQ22536. D) Calculated mean CO_2 sensitivity (Δ Hz / mmHg PCO_2) in control conditions and following SQ22536 drug application. The data presented is from 7 fibres from 4 CB preparations. Error bars indicate ± S.E.M. For B), * denotes $P < 0.05$ compared with control group; one way repeated measures ANOVA with Bonferroni post hoc analysis. For C) and D), * denotes $P < 0.05$ compared with control group; paired t-test.

6.3 Chapter synopsis and discussion

6.3.1 Summary of key findings

The main findings of the current chapter are described as follows;

- Inhibition of the extracellular adenosine generated from CD73 almost completely abolished CB basal chemoafferent activity, significantly altered the set point for hypoxia stimulus response coupling and impaired hypoxic sensitivity, but did not change the absolute peak discharge frequency induced under severe hypoxic conditions.

- The CB response to mild mitochondrial inhibition with NO_2^- was significantly attenuated by non-selective adenosine receptor antagonism. Inhibition of extracellular adenosine derived from CD73 almost completely eradicated the CB excitation induced by NO_2^- in all fibres tested.

- Hypercapnia stimulus response coupling in the CB was markedly reduced by adenosine receptor blockade and was almost entirely removed in the absence of CD73 activity. Selective inhibitory targeting of transmembrane adenylate cyclase evoked a significant reduction in the CB basal chemoafferent activity and impaired the sensory response to hypercapnia.

6.3.2 The neuromodulatory actions of extracellular adenosine derived from ecto-5'-nucleotidase on the chemoafferent discharge frequency in normoxia

Findings from this study suggest that extracellular adenosine derived specifically from ATP and CD73 activity in normoxic/hyperoxic conditions is necessary for the generation of basal chemoafferent discharge frequency in the CB. A tonic release of ATP into interstitium from type I cells has been proposed previously, based upon findings that measurable amounts of ATP can be recovered from the superfusate of the whole organ following periods of relative normoxia (10). The same study reported that inhibition of CD73 with AOPCP evoked a 2-3 fold increase in extracellular ATP concentration in the same normoxic conditions, indicative of a very high rate of basal extracellular ATP catabolism. It was observed that production of adenosine in normoxia was dependent on CD73 activity and not on the ENT transporter.

The results described in this chapter show that this pool of adenosine is of critical functional importance for the maintenance of CSN activity *in vitro*. In view of this previously uncharacterised finding it is proposed that *in vivo* maintenance of adenosinergic pathways would be central to the respiratory drive arising from the CB in arterial normoxia.

6.3.3 The impact of adenosine on carotid body stimulation by hypoxia

Adenosine formation through extracellular purine catabolism by CD73 also seems to be of functional importance in mediating the CB response to hypoxia. CD73 inhibition attenuated the chemoafferent discharge frequency during the entire response to graded hypoxia (PO_2 approximately 300–100 mmHg). The degree of inhibition was enhanced in moderate hypoxia indicating that CD73 deactivation impairs the overall CB hypoxic

sensitivity. That said, at a single level of very severe hypoxia (PO_2 approximately 60 mmHg) inhibition of CD73 did not depress the peak level of chemoafferent excitation. Collectively, these data imply that at any level of mild to moderate hypoxia, a restriction on the adenosinergic signalling pathways translates into a functional depression of the chemoafferent discharge frequency. Once a critical hypoxic threshold has been reached, this inhibition is overcome, allowing for the discharge frequency to increase, albeit at a lower PO_2. This is consistent with previous reports showing that the effect of A_2 receptor blockade was greater in mild to moderate hypoxic conditions than in a very severe hypoxic environment (10).

More detailed analysis of the parameters of the hypoxic response revealed that adenosine produced from CD73 is specifically important in setting the PO_2 threshold at which the response to hypoxia is initiated. This was demonstrated by all hypoxic response curves being ‘left shifted’ in the presence of the CD73 inhibitor AOPCP compared to their paired controls. This change in PO_2 threshold is also the most plausible explanation for the overall depression in CB hypoxic sensitivity. Whilst the initiation of the increase in discharge frequency occurred consistently at a lower PO_2 value, the actual rate of exponential increase following onset of the response was enhanced by the CD73 inhibition. The exaggerated augmentation of the exponential increase in discharge frequency produced response curves that lacked any significant gradation. This may have been because of a ‘build up’ of excitatory factors during the inhibitory phase, making the response to hypoxia more discrete rather than continuous. Taken together these data indicate that adenosine derived from CD73 is not only an important mediator in establishing the hypoxic sensitivity of the CB but its production also allows for the early phase of the chemoafferent response to hypoxia to be graded.

6.3.4 A role for adenosine derived from ecto-5'-nucleotidase on the carotid body sensory response to mild mitochondrial inhibition with nitrite

CB stimulation evoked by mitochondrial inhibition shares a number of the transduction processes known to be present in hypoxia signalling, including inhibition of TASK-like and TREK-1 conductances, Ca^{2+} influx and neurosecretion (18). It was predicted that attenuation of the adenosinergic signalling through targeting of CD73 would have a similar inhibitory action on the CB response to mitochondrial inhibition as with hypoxia. Previously, the neuromodulatory action of adenosine on the CB response to mild mitochondrial inhibition had not been studied. Chronic mild inhibition of mitochondrial complex I has been put forward as being central to the chronic increase in both CB basal activity and the response to hypoxia following CIH (313). Although the precise mechanism of NO_2^- induced mitochondrial inhibition may be different to that seen after CIH, it was used here to give a potential insight into the neuromodulatory actions of adenosine on a mild mitochondrial stimulus similar in magnitude to that observed following CIH.

Adenosine receptor antagonism and inhibition of CD73 both significantly depressed the level of chemoafferent excitation induced by NO_2^- and these findings are indicative of an important role for adenosine in mediating the CB response to mild mitochondrial inhibition. Attenuation by selective targeting of CD73 produced greater attenuation than that elicited by adenosine receptor antagonism alone. This suggests that at the dose of 8-SPT used in these studies, full antagonism of all adenosine receptors may not have been achieved or maintained throughout the entire protocols.

These findings also suggest that for chemoafferent responses to NO_2^- to be preserved, generation of adenosine from extracellular rather than intracellular ATP catabolism is of more functional significance. Pharmacological inhibition of CD73 almost completely prevented the rise in chemoafferent discharge frequency induced by NO_2^-. If adenosine produced within the type I cell and released through ENT was important in mediating CB excitability then a large component of this response would have been preserved even in the presence of CD73. Since this was not the case, it is proposed that adenosine released through ENT is not functionally significant under these circumstances. This could be confirmed in future experiments by demonstrating that the response to NO_2^- is unaffected by pharmacological blockade of ENT.

These results do support the hypothesis that adenosine generated from CD73 significantly contributes to setting the chemosensitivity of the CB to mild mitochondrial inhibition. If adenosine has a similar role in mediating CB excitability following CIH then inhibitory targeting of CD73 may be able to partially reverse the sLTF. Future experiments may aim to characterise a role for adenosinergic signalling in modifying CB hypersensitivity in animals following CIH or CHF. Therapeutically, selective targeting of the CD73 within the synapse between the type I cell and afferent neurone may be necessary because of the other numerous essential physiological actions of adenosine that are also dependent on CD73 activity. Comprehensive characterisation of the downstream effector molecules involved in CB adenosinergic neuromodulation may allow for the development of alternative pharmacological agents that selectively reduce CB chemoafferent activity without provoking systemic complications.

6.3.5 The impact of adenosine and cAMP in establishing the carotid body sensitivity to hypercapnia and other related stimuli.

Chemoafferent excitation induced by hypercapnia (80 mmHg PCO_2) was significantly diminished by adenosine receptor antagonism and almost completely abolished by inhibition of CD73 activity. The suppressing effect caused by loss of CD73 activity was greater than that of adenosine receptor blockade. Consistent with Section 6.3.4, this was indicative of a failure of 8-SPT (at the concentration used) to evoke complete adenosine receptor antagonism throughout the entire protocols. The data also implies that extracellular generation of adenosine through CD73 activity, rather than adenosine release through ENT, was necessary for coupling hypercapnia to CB chemoafferent stimulation.

The findings described in this chapter identify an essential role for endogenous CD73 generated adenosine in establishing the sensitivity of the CB to hypercapnia. This was consistent with the impact of adenosine on mediating CB responses to mitochondrial inhibitors and hypoxia. These three stimuli share many of the same chemotransduction processes including TASK-like and TREK-1 channel inhibition, Ca^{2+} influx and neurosecretion (18). Synergistic interactions between the hypercapnic and hypoxic stimuli have been described in previous reports (46, 210), and in Chapter 3 it was shown for the first time that mitochondrial inhibition with NO_2^- potentiated the chemoafferent response to hypercapnia. The precise site of interaction remains unidentified but the generation of multiplicative responses may be a consequence of an up-regulation in some or all of the processes mentioned above. Given the close association between these stimuli it is probable that adenosine establishes the sensitivity to all three by acting

on the same as yet unidentified intracellular target directly involved in one of the shared chemotransduction mechanisms.

All cloned adenosine receptors are coupled to tmACs and receptor activation induces changes in $[cAMP]_i$ (reviewed in (295).It is established that exogenous adenosine application increases the cAMP content of the intact *in vitro* CB (298). Elevations in cAMP content have also been identified in hypoxia and hypercapnia (206, 208). Inhibitory targeting of tmAC has recently been shown to attenuate CB ^{3}H-DA secretion in response to hypoxia (386). In the present investigation, the same inhibitor of tmAC (SQ22536) significantly reduced the basal chemoafferent frequency and attenuated the CB response to hypercapnia. Collectively, these data suggest that cAMP is an important intermediate downstream signalling molecule that is involved in modulating basal CB excitability and in establishing the sensitivity of the CB to hypoxia and hypercapnia.

Full characterisation of the direct and indirect intracellular targets of cAMP is yet to be fully resolved. It has been proposed that within the CB type I cell the activity of PKA and EPAC is enhanced in response to increased levels of cAMP (299, 386). Exogenous adenosine also directly attenuates the TASK-like K^+ current (299). A depression in TASK-like current has been previously implicated in both hypoxic and hypercapnic signalling in the CB. PKA phosphorylation of the TASK-like channels could potentially impair K^+ current in a similar manner to that observed in the presence of AMPK (88). Thus, the importance of adenosine derived from CD73 in mediating basal CB excitability and in establishing the CB sensitivity to hypoxia and hypercapnia may be conferred through selective attenuation of the TASK-like current. Whether PKA or EPAC directly

modifies TASK-like or other channels in the CB is unknown and may be the focus of future investigations.

In addition, the observed functional impact of adenosine on CB function may be in part due to the activation of A_{2A} receptors on the post-synaptic chemoafferent neurone (297). Post-synaptic A_{2A} mediated modification of chemoafferent neuronal activity is likely to involve alterations in $[cAMP]_i$ and activation of downstream targets such as PKA and PKC, consistent with the excitatory adenosinergic signalling pathways that have been characterised in a number of different regions in the CNS (388). The consequence of variations in $[cAMP]_i$ on post-synaptic chemoafferent neuronal excitability has not been reported in the CB and may be the subject of future studies.

6.3.6 Justification of drug concentrations

Conclusions from the present work are based on the selectivity of the drugs used. Drug types and concentrations were chosen primarily, based on previous work performed in the CB field, but also to overcome potential diffusion distance limitations known to be present in the whole intact CB preparation. A dose of 100 µM AOPCP was chosen as this concentration has been shown previously to decrease adenosine generation in both normoxic and hypoxic conditions in the intact CB preparation (11). 8-SPT was used at 300 µM, the same concentration previously reported to decrease the chemoafferent response to AICAR in the intact CB preparation (88). Importantly, in superfused arterial ring preparations, similar concentrations of 8-SPT (100–300 µM) have been demonstrated to inhibit responses to adenosine whilst allowing for responses to ATP or ATP analogues to remain intact, indicative of adenosine receptor selectivity (389, 390). SQ22536 has been used as an inhibitor of tmAC in the CB in both whole organ and cell

preparations (299, 386, 391). The concentration used in this study (200 μM) was 2-3 times greater than that used on type I cells in previous studies in order to overcome diffusion limitations. Despite this relatively high concentration, the smaller inhibitory effect of SQ22536 compared with AOPCP and 8-SPT is maybe suggestive of a degree of incomplete tmAC saturation. Given that the CB expresses mRNA for 9 isoforms of tmAC (Nunes et al, unpublished findings) it is plausible to suggest that SQ22536 may not have caused full inhibition of each tmAC isoform activated by adenosine because of varying selectivity towards each subtype. Other similar non-selective tmAC inhibitors are available and future experiments could be performed using a combination of these agents to confirm the importance of tmAC derived cAMP in modulating CB excitability.

6.3.7 Conclusions

Endogenous adenosine produced from extracellular catabolism of ATP in the presence of ecto-5'-nucleotidase (CD73) is necessary for the generation of a basal chemoafferent discharge frequency. This source of adenosine is also significant in establishing the sensitivity of the CB to mitochondrial inhibition, hypoxia and hypercapnia. The excitatory actions of adenosine are most likely to be mediated through changes in $[cAMP]_i$. Comprehensive characterisation of adenosinergic signalling pathways inherent within the CB needs to be evaluated further.

7. Overall discussion

7.1 General summary of key findings

A key finding in the studies described in this thesis is that mild mitochondrial inhibition with exogenous NO_2^- increased the sensitivity of the CB to hypoxia. An interaction of this nature has been previously uncharacterised and these results provide direct evidence showing that modification of mitochondrial function is integral to CB O_2 sensing. Subsequent studies showed that the chemoafferent response to hypoxia was almost completely abolished in CBs isolated from mice deficient in Lkb1. Lkb1 is recognised as the essential kinase required to achieve full activation of AMPK, a protein widely regarded as the sensor of cellular energy status. The observations described in this report strongly suggest that activation of the Lkb1-AMPK signalling cascade is the fundamental process that couples hypoxia induced mitochondrial inhibition with type I cell depolarisation and stimulation. A mechanism of this type unifies the two previously described ‘mitochondrial’ and ‘membrane’ hypotheses for CB hypoxia sensing.

Despite the close association between impaired cellular metabolism and CB stimulation, further observations demonstrated that the freshly isolated intact CB was not acutely sensitive to glucose deprivation. This confirms previous reports suggesting that the CB is highly unlikely to act as a physiological gluco-sensor. The ability for the CB to sustain basal chemoafferent activity in the absence of glucose was found to be dependent on the metabolism of glycogen. The chemoafferent stimulation described in response to

prolonged glucose deprivation was consistent with a time dependent run-down in glycogen and a complete inhibition of glycolysis. A significant change in the metabolic status or glycogen content of the type I cell or afferent neurone may account for the acute sensitivity to low glucose observed in other long-term culture CB preparations. These data also demonstrate, for the first time, an energy store in type I cells that might account for its ability to sustain activity during high and/or prolonged periods of stimulation. In addition, the potentially labile nature of this store, under *in vitro* conditions, might account for the qualitative discrepancies in the literature regarding the response of the CB to glucose deprivation.

The final set of experiments showed that pharmacological deactivation of CD73 led to the obliteration of basal CB chemoafferent activity, decreased the hypoxic sensitivity and completely abolished the responses to hypercapnia and mild mitochondrial inhibition. This indicates that extracellular catabolism of ATP is the main source of adenosine within the CB in normoxia and that tonic generation of adenosine acts to establish the overall sensitivity of the CB to a number of different physiological stimuli. Future development of clinical interventions targeted to selectively modify CD73 activity or adenosinergic signalling within the CB may be used to reduce the increase in chemoafferent activity associated with certain pathologies.

7.2 The carotid body mitochondrion as an acute O_2 sensor

The fundamental feature of the CB is its ability to produce functional responses to reductions in P_aO_2 before the whole body metabolism is irreversibly impaired. In this way, the CB must have a highly specialised acute O_2 sensing mechanism that is

activated before the P_aO_2 falls to levels that begin to reduce mitochondrial electron transport and ATP generation in other cell types. It is recognised that mitochondrial poisons in the absence of hypoxia, are able to evoke CB stimulation. Observations from Chapter 3 emphasised that the extent of chemoafferent excitation was closely associated with the degree of mitochondrial inhibition. More importantly, additional results showed that mild inhibition of the type I cell mitochondria using exogenous NO_2^- sensitised the CB to hypoxia. In the presence of NO_2^-, functional chemoafferent responses to hypoxia were initiated at higher PO_2 values. When applied simultaneously, these two stimuli generated multiplicative, rather than additive responses indicative of a significant degree of stimulus interaction. In view of these findings it is proposed that inhibition of type I cell mitochondrial function is the fundamental mechanism required for initiation of the CB response to hypoxia.

The type I cell mitochondria appear to have a uniquely low affinity for O_2. Previous experiments have detected a degree of mitochondrial depolarisation and attenuation of electron transport at PO_2 levels as high as 40 mmHg (4, 56). This suggests that type I cell mitochondrial function will start to be impaired at PO_2s significantly above those that would begin to reduce mitochondrial activity in other cell types. This feature is highly significant because it identifies a precise mechanism by which the type I cell can be exquisitively sensitive to relatively small reductions in PO_2.

But why does the type I cell mitochondrion have such a low affinity for O_2? It has been previously reported that cytochrome a_3 in complex IV in the CB was reduced at PO_2 values between 40 and 80 mmHg (3). This perhaps indicates that the CB possesses a unique isoform of cytochrome a_3 with a configuration that makes binding of O_2 to the

Cu_B/haem a_3 binuclear centre much less favourable. Alternatively, another endogenously produced factor may act to impair O_2 binding at this site. Exogenous NO_2^- was used in this study because of its unique potential to generate NO locally at the mitochondria. Observations from Chapter 3 were consistent with the excitatory actions of NO_2^- being a consequence of initial NO formation and impairment of mitochondrial function. This implies that if enough endogenous NO is produced locally at the mitochondria (either through NOS or NO_2^-) then it may act to reduce the affinity of cytochrome a_3 for O_2 in complex IV. However, a physiological role for endogenous NO in modifying type I cell mitochondrial activity is yet to be confirmed.

7.3 Lkb1-AMPK signalling as a fundamental process that couples hypoxia to chemoafferent excitation

Experiments performed in Chapter 4 aimed to characterise a component of the signalling process that couples hypoxia induced mitochondrial impairment with chemoafferent stimulation. It was observed that conditional deletion of Lkb1 almost completely ablated the CB chemoafferent response to hypoxia. This suggests that Lkb1 is necessary for eliciting CB activation in hypoxia.

The best characterised function of Lkb1 is its ability to phosphorylate the Thr 172 residue in the activation loop on the catalytic α subunit of AMPK, thereby promoting activation. Lkb1 mediated phosphorylation is recognised as the essential step required for complete activation of AMPK. AMPK is widely regarded as the global sensor of cellular energy status because its activity is tightly regulated by the relative intracellular concentration of adenosine nucleotides, and in particular the AMP:ATP ratio. In the CB,

impairment of mitochondrial electron transport in hypoxia has the potential to cause a reduction in $[ATP]_i$, through decreased ATP synthesis, and an increase in $[AMP]_i$, subsequent to activation of adenylate kinase. It is proposed that, as long as Lkb1 is functional, an increase in the AMP:ATP ratio would account for activation of AMPK following a depression in mitochondrial energy metabolism in hypoxia.

Previous studies have shown that AMPK activation with AICAR stimulates CB chemoafferent activity and this effect is associated with type I cell depolarisation and Ca^{2+} influx (5, 88). The direct targets of AMPK in the type I cell appear to be a number of different K^+ channels including TASK, TREK-1 and BK_{Ca}, all of which have been implicated in CB O_2 sensing (5, 29, 88). Proposed deactivation of AMPK by Compound C is known to partially attenuate the chemoafferent response to hypoxia by approximately 50% (88). The results presented in this thesis (utilising genetic technology to entirely diminish Lkb1-AMPK signalling) are more conclusive and indicate that activation of AMPK by Lkb1 phosphorylation is the essential process that couples mitochondrial induced metabolic stress with CB activation in hypoxia.

7.4 The importance of glycogen metabolism in the carotid body upon exposure to glucose deprivation

The findings described in Chapter 5 clearly establish that the freshly isolated intact CB is not acutely sensitive to glucose deprivation. A significant chemoafferent response was recorded, but only after approximately 20-40 minutes of exposure to the glucose free stimulus. In preliminary experiments, isolated type I cells did not exhibit any excitation in

response to glucose deprivation even after 40 minutes of exposure. It is therefore proposed that the CB cannot function as a physiological low glucose sensor *in vivo*.

The preservation of normal chemoafferent activity in glucose free conditions suggests that the CB is able to maintain metabolic status in the absence of extracellular substrate delivery. This is based on findings from previous chapters clearly suggesting that a reduction in mitochondrial activity and cellular energy status would lead directly to Lkb1-AMPK activation and CB excitation. The reason for this apparent lack of metabolic stress in glucose free conditions was shown to be dependent on the metabolism of glycogen, allowing for the maintenance of glycolysis and sufficient acetyl CoA delivery into the mitochondria. Pharmacological inhibition of glycogen phosphorylase activity or proposed AMP mediated depletion of glycogen during periods of severe hypoxia, both significantly reduced the time taken for the CB to respond to glucose deprivation. The increase in chemoafferent stimulation following prolonged glucose free exposure was rapidly reversed by only 1 mM glucose or by addition of lactate and pyruvate. This supported the idea that the CB was only stimulated in response to a depleted cellular energy status following complete inhibition of glycolysis and exhaustion of glycogen reserves.

These findings are in direct contrast with others that have shown a direct sensitivity of the CB to low glucose (246, 248). The reason for these clear discrepancies may be dependent on a variation in metabolic status of the CB tissue used across all of the different *in vitro* preparations. In the investigations performed by Pardal et al. (246) and Zhang et al. (248) the CB was only isolated up to 5 minutes after decapitation. This is likely to have subjected the CB tissue to a period of severe ischaemia and hypoxia and

consequentially may have significantly depleted glycogen stores. This may have made the CB much more sensitive to a reduction in extracellular glucose concentration. Furthermore, a sensitivity to low glucose reported in these same studies was only observed in CB tissue following periods of long term (between 3 and 7 days) hyperoxic incubation. The evidence presented in Chapter 5 identified a significant reduction in the time taken to respond to glucose deprivation 18-24 hours after removal of the CB from the animal. A reason for this change remained unidentified although it appeared not to be dependent on hyperoxic induced ROS generation. Nevertheless, long term incubation does seem to be important in altering the CB sensitivity to low glucose and this may significantly account for the findings reported in other investigations.

7.5 Adenosine derived from ecto-5'-nucleotidase as an important neuromodulator in the carotid body

The main focus of the final results chapter was to examine the potential impact of adenosine on CB function. ATP is not only a metabolic signalling molecule in the CB, it is also an important excitatory neurotransmitter that evokes depolarisation in the adjacent chemoafferent neurone leading to post-synaptic action potential generation. Following release, extracellular catabolism of ATP in the presence of CD39 and CD73 has the added potential to rapidly form a significant pool of extracellular adenosine.

Initial studies found that pharmacological inhibition of CD73 failed to depress the chemoafferent responses to a single level of very severe hypoxia. However, more detailed analysis of graded chemoafferent hypoxic response curves revealed that deactivation of CD73 significantly impaired CB hypoxic sensitivity. This was achieved by

altering the threshold required for hypoxic response initiation. In other words the PO_2 at which the CB began to respond was lower in the absence of adenosine derived from CD73. These observations provide direct evidence supporting the proposal that adenosine generated from extracellular catabolism of ATP in the presence of CD73 has an important role in establishing the sensitivity of the CB to hypoxia.

The impact of CD73 antagonism on basal CB activity and on responses to more moderate stimuli was even more striking. The basal chemoafferent discharge frequency was obliterated following inhibition of CD73. The sensory responses to hypercapnia and a moderate dose of NO_2^- were almost entirely abolished. This establishes that adenosine is tonically produced from CD73 and is essential in generating the basal chemoafferent discharge frequency. Adenosine also seems to have a critical functional role in setting the threshold at which the CB begins to respond to a number of different stimuli.

Adenosine is likely to act through stimulation of A_2 receptors leading to an increase in cAMP production from tmACs. Similar inhibitory effects on CB basal activity and on responses to hypercapnia were observed in the presence of adenosine receptor blockade and upon non-specific antagonism of tmACs. Previous studies have also reported that the same inhibitor of tmAC (SQ22536) attenuated the CB response to a single level of hypoxia (386). In view of these observations, it is proposed that cAMP has a vital role in regulating the basal CB excitability and in part controls the stimulus intensity threshold required for response initiation. The downstream targets of cAMP were not identified in this project. Intermediate activation of PKA and/or EPAC may be of significance given their established association with cAMP. However, the ultimate target

is likely to be common to the pathways involved in sensing hypoxia and hypercapnia and additionally, the target must be active under basal conditions. Modification of adenosinergic signalling or of the as yet unidentified downstream targets of cAMP may prove to be important in reversing the hyperexcitability of the CB in CHF or following CIH.

7.6 Key method limitations

7.6.1 Carotid body isolation

All of the experiments described in this thesis were performed *in vitro,* on CB tissue or cells following surgical isolation of the intact organ from the animal. In all surgeries, the CB was harvested only after the CCA had been clamped. The organ would have been exposed to a short period of ischaemia, hypoxia and potentially acidosis, all likely to have induced intense CB stimulation. This may have evoked an irreversible depletion of neurotransmitter stores and glycogen content and in some cases may have brought about permanent cell damage or even necrosis. To limit the time of ischaemia, CB tissue was isolated as rapidly as possible and within 30 seconds of clamping of the CCA. However, it is predicted that chemoafferent responses recorded *in vitro* would not have absolutely corresponded to the equivalent responses had they been measured *in vivo*.

7.6.2 The *in vitro* superfused whole organ carotid body preparation

The majority of studies in this report were performed using the *in vitro* superfused intact carotid body preparation. This is a widely used preparation and it allows for the maintenance of structural organ integrity. However, gases, substrates or drugs must

diffuse or be transported across the whole organ from the external superfusate in order to reach the cellular targets within the tissue. As a consequence of potential diffusion or transport limitations, superfusate recordings of PO_2s, PCO_2s and drug concentrations may not have accurately reflected the actual levels that the individual type I cells were exposed to.

It has been estimated that in the intact rat CB preparation *in vitro* (approximately 200-300 µm diameter), the maximum difference between the superfusate PO_2 at the CB surface and the tissue PO_2 at the centre of the CB was approximately 80 mmHg (48). Therefore, the actual PO_2 at the level of a type I cell cluster may be up to 80 mmHg lower than the recorded superfusate PO_2. Impairment of O_2 diffusion may be amplified further depending on the amount of connective tissue and fat that surrounds the surface of the CB capsule. In order to ensure adequate metabolism for all cells, basal chemoafferent discharge frequency (unless stated otherwise, as in Chapter 5) was determined at a superfusate PO_2 of 300 mmHg in the rat CB and 200 mmHg in the mouse CB (assumed to have a smaller diameter). There is a strong possibility that a number of cells would have been exposed to hyperoxia under these conditions. The term ‘basal discharge frequency’ described in this thesis is only used as a representation of spontaneous CB activity and should not be thought of as the absolute frequency that would be expected in normoxia. However, because of the known shape of the chemoafferent PO_2 response curve (see Chapter 2), these values are hypothesised as being similar.

In all experiments described in this thesis, the superfusate PO_2 at which the chemoafferent response to hypoxia was initiated was consistent with the range reported

in previous studies using the *in vitro* mouse (132) and rat (46) CB preparations. The hypoxic response curves generated from these superfused *in vitro* CB preparations seem to be relatively ‘right shifted’ compared to those obtained *in vivo*. This apparent discrepancy is most likely a consequence of the PO_2 diffusion limitations present across the whole organ.

The position of a single chemoafferent response curve in relation to the superfusate PO_2 will depend on the relative depth of the type I cell cluster and chemoafferent neurone within the CB. This ensures that for a single CB preparation, PO_2 responses curves may be observed over a wide range of different PO_2s. Therefore, for the majority of studies, the PO_2 response curves calculated in the presence and absence of exogenous drug applications were made from recordings of the same chemoafferent fibre (innervating the same type I cluster). This ensured that data was ‘paired’ and that the conclusions were based on the impact of the drug on CB function and not on inconsistencies in the PO_2 across the whole tissue.

A number of the conclusions formed in this thesis are largely based on the action of exogenous pharmacological agents. Drug types and concentrations were chosen primarily based on precedents established in previous work performed in the CB field. In some cases higher concentrations were used than have been reported in the literature. This was to overcome the potential diffusion and transport limitations across the CB tissue. The absolute drug concentration that type I cells were exposed to when the chemoafferent discharge frequency was being recorded was unable to be determined. Therefore, these results should be viewed with some caution because of the potential partial or non-selective actions of the agents used.

7.7 Future experiments

7.7.1 Characterisation of the carotid body mitochondrial complex IV

In view of the results from this thesis and others, it is suggested that O_2 in the CB type I cell is detected at the level of the mitochondria and specifically within complex IV. The unique low affinity binding of O_2 to complex IV could be related to the expression of cell type specific mitochondrial isozymes of the O_2 sensitive proteins. A key experiment would be to characterise the phenotype of the type I cell mitochondria. This could be done by initially extracting mRNA from dissociated CB type I cells. As comparators, mRNA would be extracted from rat liver (often used for mitochondrial studies, and represents a 'standard' and well-defined mitochondrial phenotype) and adrenal medullary cells. These mRNA samples would be subjected to transcriptomic analysis. Gene expression data would be analysed and based on databases of mitochondrial proteins altered isozyme mRNA expression in type I cells in comparison to liver and adrenal medullary cells could be detected.

Data from this transcriptomic study would provide a 'model' isozyme profile of the electron transport chain (and in particular complex IV) in type I cells compared to both liver and adrenal medullary cells. Then, using siRNA and expression constructs, the adrenal medullary isozyme expression profile would be altered to mimic the type I cell, starting with single component changes in complex IV. Having confirmed the correct profile, changes in the mitochondrial function and excitability of the adrenal medullary cells in response to O_2 would be examined to see whether this had been altered to mimic the type I cell phenotype.

Alternatively, the low affinity binding of O_2 in complex IV may be mediated by some other extra-mitochondrial factor. A potential candidate identified from studies in this thesis is NO. Whether or not endogenous NO sets the mitochondrial O_2 sensitivity remains to be determined. Interestingly, hypoxic ventilatory responses appear to be attenuated in NOS-3 deficient mice, suggestive of a reduction in CB hypoxic sensitivity. Future studies using isolated CB tissue could examine whether mitochondrial sensitivity to O_2 is altered in these NOS-3 KO mice and if so, is this coupled to an attenuation of the functional chemoafferent response to hypoxia? Conditional deletion of NOS-3 in cells expressing TH would be achieved by use of Cre-Lox recombination technology similar to that described in Chapter 4. This would eliminate the potential confounding complication of chronic local vasoconstriction and chronic hypoxia from birth, a condition also known to impair acute CB hypoxic sensitivity.

7.7.2 Confirmation of AMPK as the link between the mitochondria and K^+ channels

The data presented in this thesis strongly supports the hypothesis that activation of Lkb1-AMPK signalling subsequent to the hypoxia induced impairment of mitochondrial energy metabolism couples directly to CB stimulation. This was based on observations from experiments identifying a powerful attenuation of hypoxic sensitivity in CBs deficient in Lkb1, the essential kinase required to activate AMPK. However, since the STRAD-Lkb1-MO25 complex is known to phosphorylate a total of 13 kinases (348), it is possible that the loss of hypoxic sensitivity was achieved through the diminished activity of any one of these other proteins.

To confirm the role of AMPK in O_2 sensing, similar experiments to those described in Chapter 4 should be performed on CBs isolated from mice deficient in AMPK. Total global AMPK deficiency is embryonic lethal and so again the use of Cre-Lox recombination technology would be used to conditionally delete AMPK expression in cells only expressing TH. Single KO of the α1 subunit may not be sufficient to completely obliterate AMPK activity because of the potential for compensatory up-regulation of the α2 subunit. Development of a conditional α1, α2 double AMPK KO mouse model would be required. In addition to recordings of CB chemoafferent activity, measurements of whole animal ventilation and type I cell $[Ca^{2+}]_i$ elevations in response to hypoxia could be made to further confirm the importance of AMPK in CB O_2 sensing. Techniques required for the successful generation of the conditional α1, α2 double AMPK KO mouse are currently in development.

7.7.3 Identification of a molecular mechanism involved in carotid body plasticity

Both CHF and CIH have been shown to alter the O_2 sensitivity of type I cells. This could be due to transcriptional modifications such as changes in isozyme expression or post-translational mechanisms, such as protein S-nitrosation or carbonylation. Evidence suggests that a reduction in mitochondrial complex I activity subsequent to ROS generation from NOX-2 may underpin this CB hyperexcitability following CIH. A similar mechanism is likely to exist in the type I cell during CHF. Importantly, this tonic deactivation of complex I may induce chronic cellular metabolic stress leading to a chronic increase in the expression or activity of Lkb1-AMPK signalling. Experiments performed on type I cells or CBs from mice deficient in Lkb1 or AMPK following pre-conditioning with CIH would demonstrate whether Lkb1-AMPK activation is necessary to

induce sLTF. If this is found to be the case then inhibitory targeting of Lkb1-AMPK may act to attenuate CB hypersensitivity in CHF or following CIH.

7.7.4 Characterising the downstream targets of adenosine and cAMP in the carotid body type I cell

The results described in Chapter 6 suggest that adenosine is an important excitatory neuromodulator of CB chemoafferent activity. The impact of adenosine on type I cell function is predicted to be mediated through changes in cellular $[cAMP]_i$. All of the downstream targets of cAMP are yet to be comprehensively characterised in the type I cell. Two intermediate effector candidates are PKA and EPAC, both known to be stimulated by cAMP. Initially, inhibitors of PKA and/or EPAC would be used to see if they mimicked the inhibitory effect of CD73 antagonism on CB chemoafferent activity, thereby resolving whether PKA or EPAC were involved in establishing the CB sensitivity to physiological stimuli. Potentially PKA could phosphorylate a number of different proteins or channels in the CB including TASK-1, TASK-3, TREK-1, or BK_{Ca}. Western blot analysis using phosphorylation specific antibodies could be implemented to identify these potential modifications. Furthermore, pharmacological inhibition of PKA in type I cells could be used to identify whether phosphorylation of any of these channels directly impairs their K^+ conductance. Characterisation of a PKA mediated attenuation of the background K^+ current in normoxia may explain how adenosine is able to regulate basal chemoafferent activity and establish the threshold at which the CB begins to respond to a number of different physiological stimuli.

7.8 Concluding remarks

Augmentation of the sensory chemoafferent activity originating in the CB both under basal conditions and in response to hypoxia is becoming more apparent in a wide number of pathologies including SDB, CHF and essential/spontaneous hypertension (2). Up-regulation of the downstream reflex pathways tightly associated with CB stimulation in these disease states leads to a chronic increase in sympatho-adrenal activation, an elevation in baseline vascular tone and the progression of hypertension (392). Selective targeting of the CB using pharmacological agents to reduce chemoafferent outflow may therefore be crucial for restricting the development of hypertension and cardiovascular complications in these patient populations.

The development of therapeutic agents for use in clinical practice has, however, been limited due to the fact that the identity of the type I cell specific O_2 sensor and its associated downstream effectors have remained elusive. The primary experiments in the present study were designed to generate findings that advance the understanding of the precise mechanisms underpinning CB activation by hypoxia.

The results presented in this thesis demonstrate that a reduction in type I cell mitochondrial electron transport by NO_2^- directly enhances the sensitivity of the CB to hypoxia. These findings provide evidence suggesting that mitochondrial function lies at the centre of CB O_2 sensing. More specifically the results strengthen the hypothesis that the first step in the CB hypoxia stimulus response coupling process is a reduction in mitochondrial energy respiration following an impaired binding of O_2 to the Cu_B / haem a_3 binuclear centre within complex IV. Further studies indicate that activation of the Lkb1-AMPK signalling pathway (subsequent to an increase in the AMP:ATP ratio)

appears to be the key step in linking a decrease in mitochondrial function with type I cell stimulation in hypoxia. A degree of Lkb1 mediated phosphorylation of AMPK in normoxia also seems to be necessary for the generation of basal chemoafferent discharge frequency. It is predicted that future development of therapeutic agents able to selectively target and deactivate Lkb1 or AMPK in the type I cell may prove to be clinically important in reducing the augmented chemoafferent activity in certain pathologies.

In addition to hypoxia, the CB may be stimulated by a number of other external stimuli such as hypercapnia and acidosis (393). Although there is strong evidence implying that the CB is involved in systemic glucose homeostasis, the findings described in this thesis indicate a lack of intrinsic low glucose sensitivity in the type I cell. The maintenance of cellular energy status in the complete absence of external glucose consumption seems to be achieved by the metabolism of stored glycogen. This raises the intriguing question as to what factor, released as a consequence of systemic hypoglycaemia, stimulates the CB, thereby promoting the downstream reflex increase in blood glucose. This may be the focus of future investigations and could be of significant clinical importance given the rapid emergence of diseases associated with dysfunctional systemic glucose regulation.

Chemoafferent signalling into the NTS may also be modified by a host of different neurotransmitters and neuromodulators. The final chapter in this thesis identifies an important and novel functional role for endogenous adenosine, derived specifically from extracellular catabolism of ATP, in mediating basal chemoafferent outflow and in establishing the CB sensitivity to multiple related stimuli. The results suggest that the tonic production of adenosine in this manner acts to prime the CB to subsequent

stimulation. Future comprehensive characterisation of the adenosinergic pathways present in the CB may allow for the development of pharmacological agents capable of suppressing the increase in chemoafferent activity in patients with SDB, CHF and spontaneous/essential hypertension without impacting on other organ systems. All of the key findings described in this thesis are summarised in Figure 7.1.

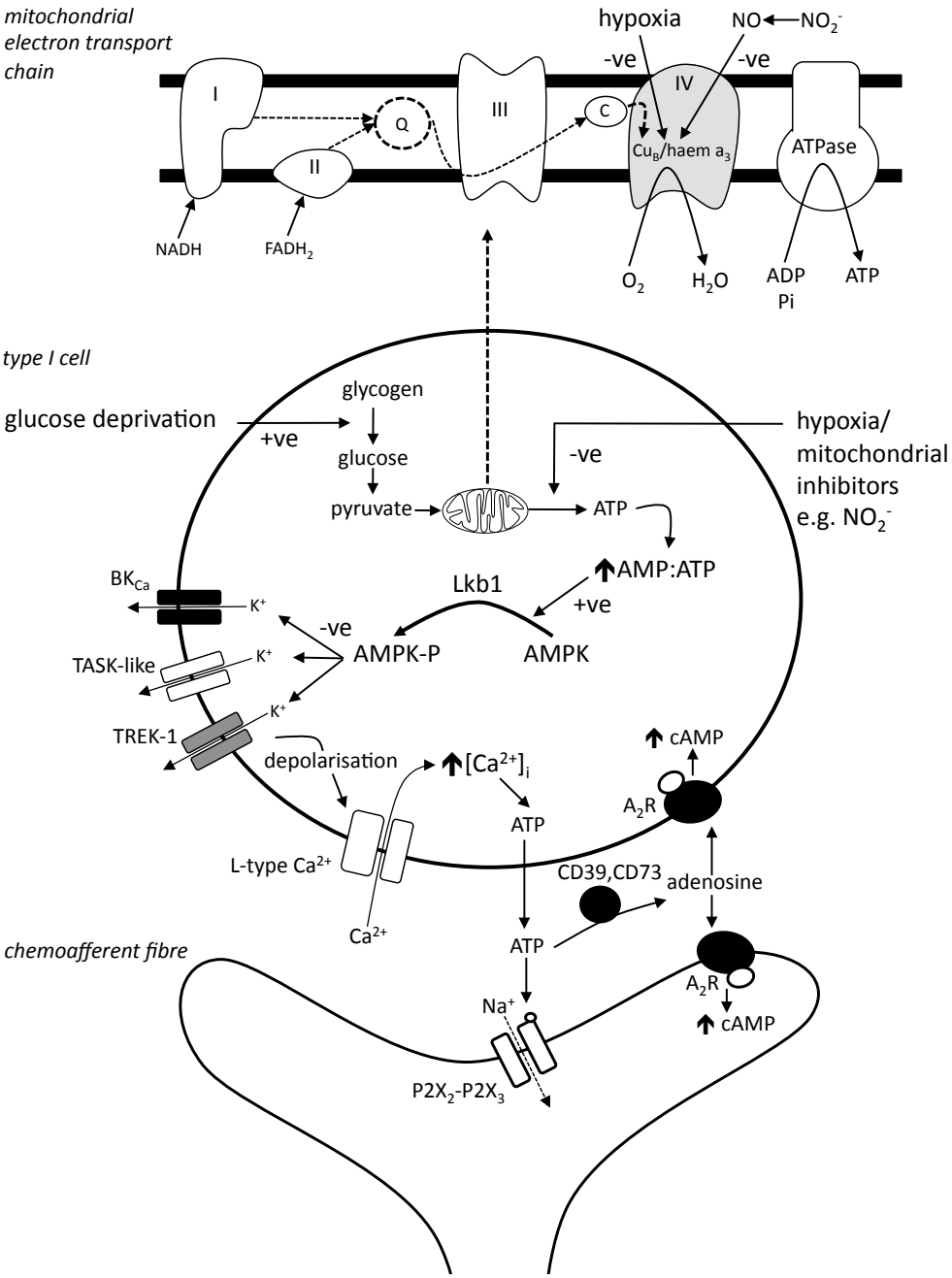
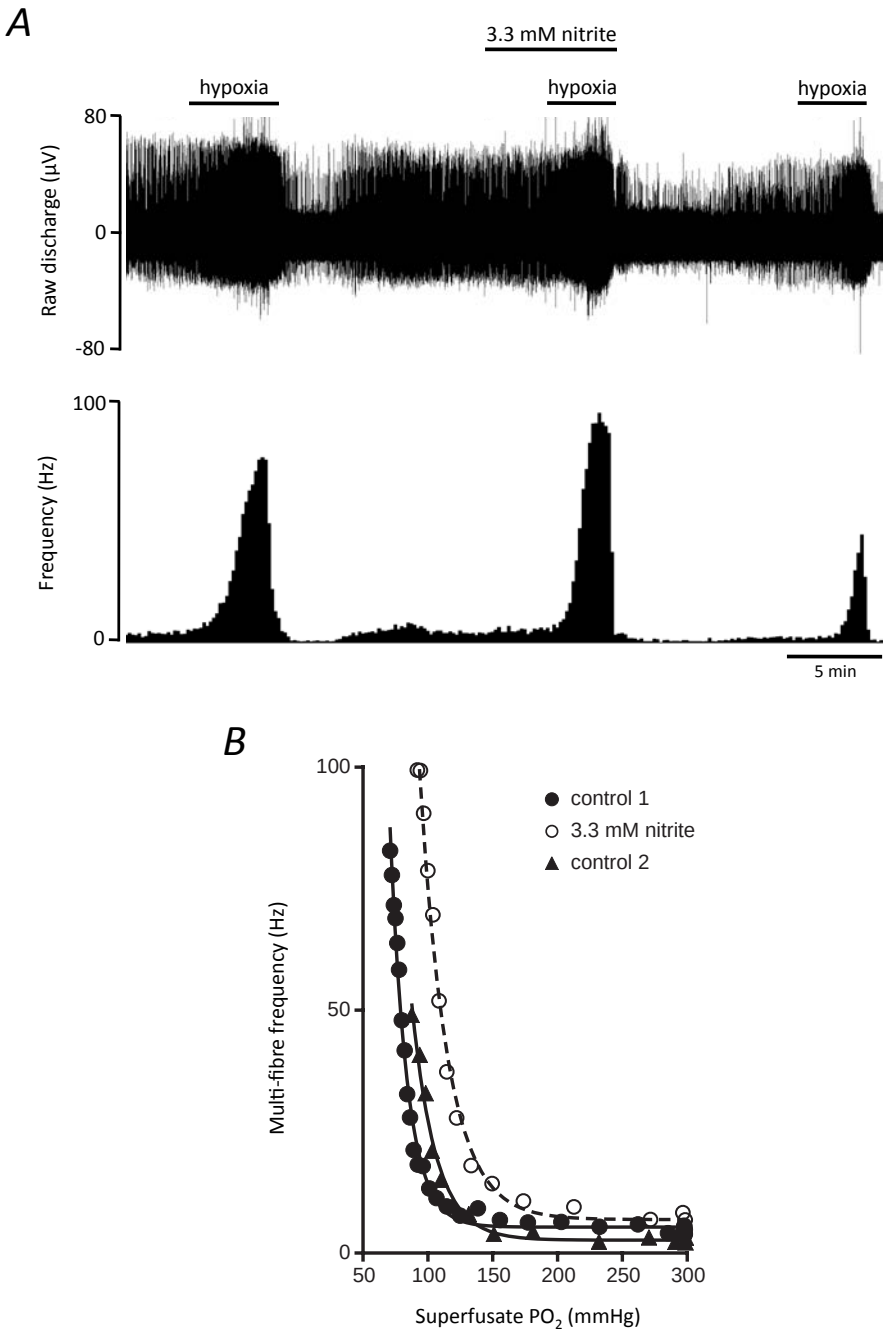
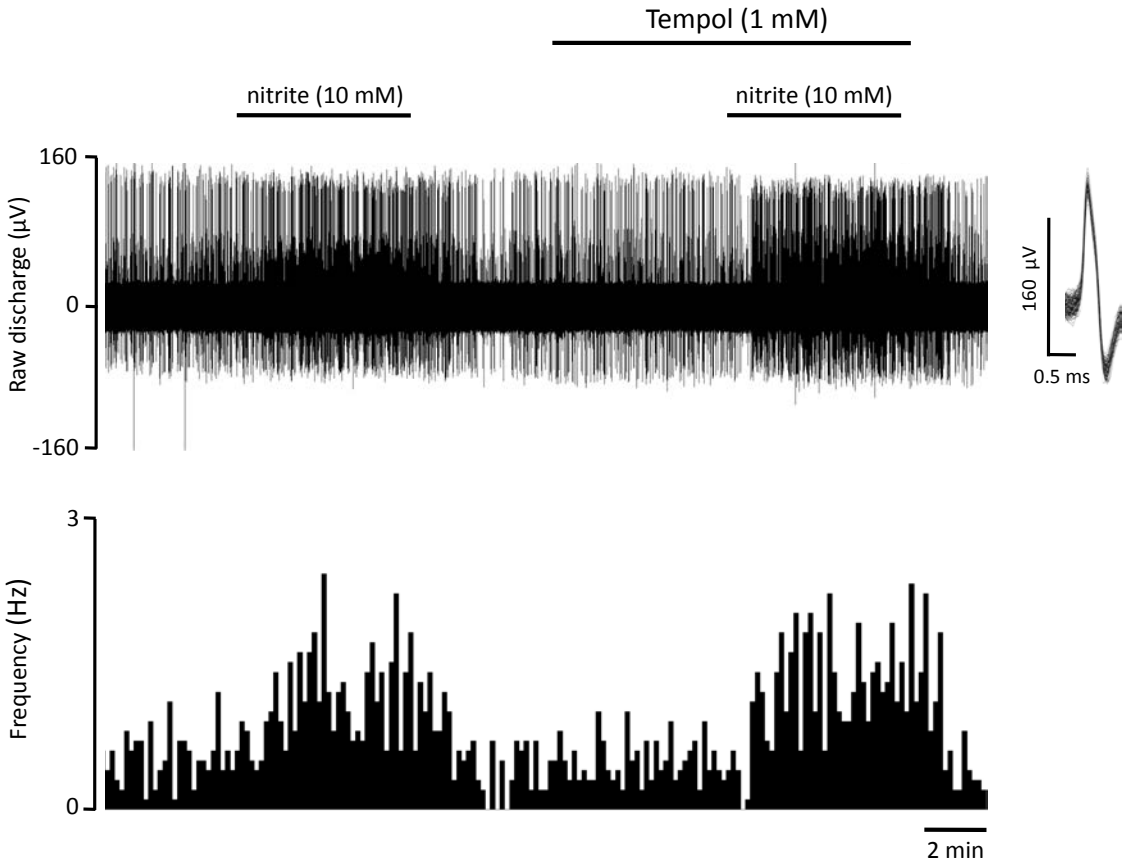


Figure 7.1 Summary of key findings.
Carotid body (CB) activation is tightly coupled to a decrease in cellular energy status. In hypoxia, an increase in the AMP:ATP ratio enhances AMPK phosphorylation by Lkb1 thereby activating downstream chemotransduction processes necessary to elevate the chemoafferent fibre discharge frequency. Type I cell mitochondrial function can be impaired by NO generated directly from NO_2^-. Mild mitochondrial inhibition with NO_2^- enhances the sensitivity of the CB to hypoxia. This suggests that the elevation in the AMP:ATP ratio required to stimulate Lkb1-AMPK in hypoxia is a secondary consequence of a reduction in mitochondrial electron transport and ATP synthesis. In response to glucose deprivation, metabolism of glycogen acts to maintain substrate delivery into the mitochondria allowing for sufficient ATP synthesis to preserve the cellular energy status. Therefore, glucose deprivation does not acutely activate the CB. ATP released as an excitatory neurotransmitter in normoxia and hypoxia is metabolised by CD39 and CD73 and leads to the generation of synaptic adenosine. This 'pool' of adenosine has a crucial function in establishing the basal chemoafferent discharge frequency and the sensitivity of the CB to stimulation by hypoxia, hypercapnia and mitochondrial inhibitors. The impact of adenosine is mediated through A_2 receptors and an increase in pre- or post-synaptic cAMP concentration.

Appendix I: Supplementary data



Supplementary figure 1 The excitatory impact of nitrite on augmenting the chemoafferent response to hypoxia in a single fibre is also apparent in a multiple fibre preparation when discharge frequency is collated.
A) An example raw trace taken from a multiple fibre recording showing the effect of 3.3 mM nitrite (NO_2^-) on the chemoafferent frequency response to graded hypoxia. Raw discharge is shown (upper) along with frequency histograms (lower) grouped in 10 s intervals. B) The characteristic 'right shift' induced by 3.3 mM NO_2^- on the chemoafferent hypoxic response curve calculated from single fibre recordings is also evident when the discharge frequency is collated from multiple fibres within the same preparation.



Supplementary figure 2 Pharmacological scavenging of superoxide (O_2^-) does not reduce the chemoafferent response to nitrite.
An example trace showing the absence of an effect of the superoxide (O_2^-) scavenger Tempol (1 mM) on the chemoafferent frequency response to 10 mM nitrite (NO_2^-). Raw discharge is shown (upper) along with frequency histograms (lower) grouped in 10 s intervals. Overdrawn action potentials are shown inset to demonstrate the single fibre discrimination from which the frequency was taken.

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